AD_____

GRANT NUMBER DAMD17-96-1-6158

TITLE: Regulation of Neu Signaling in Breast Cancer

PRINCIPAL INVESTIGATOR: Frank Jones, Ph.D.
David F. Stern, Ph.D.

CONTRACTING ORGANIZATION: Yale University School of Medicine
New Haven, CT 06520-8047

REPORT DATE: July 1999

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual Summary (1 Jul 96 - 30 Jun 99)

4. TITLE AND SUBTITLE
Regulation of Neu Signaling in Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6158

6. AUTHOR(S)
Frank Jones, Ph.D.
David F. Stern, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University School of Medicine
New Haven, CT 06520-8047

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Signaling by members of the epidermal growth factor receptor (EGFR) family plays an important role in breast development and breast cancer. Earlier work suggested that one of these receptors, ErbB4, is coupled to unique responses in this tissue. To determine the function of ErbB4 signaling in the normal mouse mammary gland, we inactivated ErbB4 signaling by expressing a carboxyl-terminally deleted dominant negative allele of *ErbB4* (*ErbB4ΔIC*) as a transgene in the mammary gland. Despite the expression of *ErbB4ΔIC* from puberty through later stages of mammary development, an *ErbB4ΔIC*-specific phenotype was not observed until mid-lactation. At 12 days post-partum, lobuloalveoli expressing ErbB4ΔIC protein were condensed and lacked normal lumenal lactation products. In these lobuloalveoli, β-casein mRNA, detected by in situ hybridization, was normal. However, whey acidic protein mRNA was reduced, and α-lactalbumin mRNA was undetectable. Stat5 expression was detected by immunohistochemistry in ErbB4ΔIC-expressing tissue. However, Stat5 was not phosphorylated at Y694 and was, therefore, probably inactive. When expressed transiently in 293T cells, ErbB4 induced phosphorylation of Stat5. This phosphorylation required an intact Stat5 SH2 domain. In summary, my results demonstrate that ErbB4 signaling is necessary for mammary terminal differentiation and Stat5 activation at mid-lactation.

14. SUBJECT TERMS Breast Cancer

15. NUMBER OF PAGES
29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

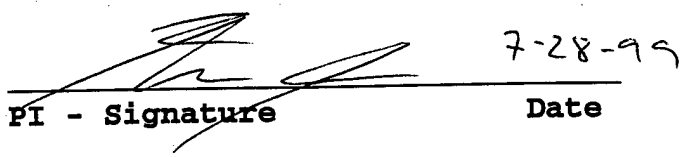
__✓__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature Date

Table of Contents

Introduction

The receptor tyrosine kinase ErbB2/HER2/Neu (referred to here as ErbB2) is amplified and overexpressed in 15-40% of human breast cancers (reviewed in: Hynes and Stern 1994). In phase III clinical trials of a recombinant humanized anti-ErbB2 monoclonal antibody, targeting of breast cancer cells overexpressing ErbB2 results in growth inhibition and regression of tumors (reviewed in: Nass, Hahm et al. 1998). These results demonstrate that ErbB2 will be a valuable target for therapeutic treatment of breast cancer patients. Recently, we have published results demonstrating ErbB2's role in mammary differentiation and lactation. ErbB2 signaling pathways coupled to mammary differentiation could be marshaled to antagonize the mitogenic effect of activated ErbB2 within an aggressively proliferating tumor. To this end, I will describe results suggesting that ErbB4 is also associated with mammary differentiation. These latter results support a model for breast cancer where ErbB4 may act as a tumor suppressor in ErbB2 overexpressing tumors.

Body

In my previous annual report I described the generation and initial characterization of transgenic mice expressing a dominant negative mutant form of ErbB4 (ErbB4ΔIC) during mammary gland development. This strategy was used to inactivate normal ErbB4 signaling during development. Preliminary data demonstrated expression of ErbB4ΔIC throughout mammary development, however a mammary phenotype was not described.

ErbB4ΔIC protein expression is associated with condensed lobuloalveoli during lactation To determine the effects of ErbB4ΔIC expression on female mammary gland development, wholemounts and histological sections were examined from virgin mice at 3, 5, 6, 8, 10, and 19 weeks of age; during early (12 days) mid (16 days) and late (19 days) pregnancy, after parturition at days 3, 6, 9, 12, 15, or 18, and 2-4 days after weaning. At least three mice were analyzed at each time point. Despite the extensive time frame of transgene expression the only identifiable phenotypes were detected on day 12 post-partum. The fat-pad of a non-transgenic mouse at 12 days post-partum is completely invested with engorged lobuloalveoli displacing stromal adipose cells. Secretory activity is demonstrated by lumens lined with protruding secretory epithelium (Fig. 1A, arrow). Engorged active secretory lobuloalveoli were also observed in ErbB4ΔIC-expressing mice at 12 days post-partum (Fig. 1B, arrow). In some transgenic mice (3 out of 5 examined), however, a subpopulation of lobuloalveoli failed to expand and contained an unusually high level of lumenal secretory lipids (Fig. 2B, asterisk). Adipose cells were still abundant in this region of the mammary gland fat-pad. The condensed lobuloalveoli resembled undifferentiated lobuloalveoli that normally predominant during late pregnancy. I next used anti-Flag immunohistochemistry to determine if the condensed lobuloalveoli expressed the Flag-tagged *ErbB4ΔIC* transgene. Intense cytoplasmic immunostaining of epithelium within condensed lobuloalveoli was observed (Fig. 1D, asterisks). Anti-Flag immunostaining was not observed in distended lobuloalveoli in the same tissue sections (Fig. 1D, arrow).

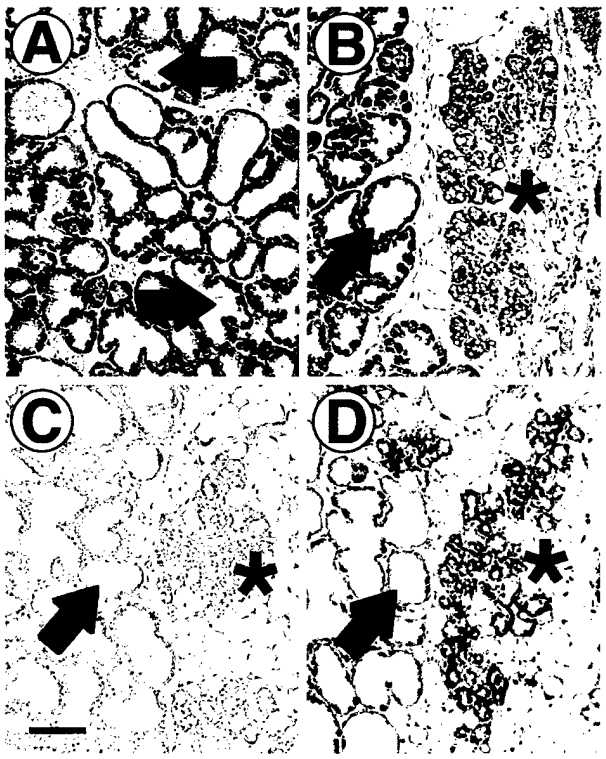


Figure 1. Immunohistochemical detection of ErbB4ΔIC protein in the mammary gland at 12 days post-partum. Paraffin-embedded section from a 12 day post-partum non-transgenic sibling control stained with hematoxylin/eosin (A). Sequential sections from a 12 day post-partum mammary gland from an ErbB4ΔIC-expressing mouse were stained with hematoxylin/eosin (B), or stained by immunohistochemistry with rabbit IgG control antibody (C), or a rabbit anti-Flag antibody (D). Expanded secretory lobuloalveoli are indicated by arrows, and condensed atypical lobuloalveoli are indicated by asterisks. Bar in C = 100 μm.

Although the alveolar condensation associated with high ErbB4ΔIC expression might be caused by selective growth inhibition or apoptosis, neither BrdU incorporation experiments, nor TUNEL analysis revealed differences between the phenotypically normal and condensed lobuloalveolar

populations in ErbB4ΔIC animals (data not shown). These results suggest instead that ErbB4ΔIC-expression inhibits normal lobuloalveolar development and function at 12 days post-partum.

ErbB4ΔIC expression inhibits milk-gene expression ErbB4ΔIC expression at 12 days post-partum impaired lobuloalveolar development, resulting in condensed alveolar structures with pronounced lipid secretory activity. These structures resembled normal undifferentiated lobuloalveoli observed at late pregnancy and parturition. To determine if the ErbB4ΔIC-expressing lobuloalveoli were lactationally active, I performed in situ hybridization using antisense riboprobes specific for the milk genes β-casein, whey acidic protein (WAP), and α-lactalbumin. Serial paraffin sections containing both normal expanded lobuloalveolar structures and condensed lobuloalveoli were examined (Fig. 2A, arrow and asterisks, respectively). ErbB4ΔIC expression within condensed lobuloalveoli was confirmed by anti-Flag immunohistochemistry (Fig. 3B, asterisks). The sense probes for β-casein, WAP, and α-lactalbumin yielded similar levels of background hybridization in both expanded and condensed lobuloalveoli (Fig. 2C, E, and G, arrows and asterisks, respectively). With antisense probe, equivalent high levels of β-casein RNA expression was observed in both the normal and ErbB4ΔIC-expressing lobuloalveoli (Fig. 2D, arrow and asterisks, respectively). However, the ErbB4ΔIC-expressing lobuloalveoli showed a moderate diminution in WAP expression (Fig. 2F). Strikingly, α-lactalbumin expression was reduced to sense probe background levels in condensed areas, but not in normal areas of the same section (Fig. 2H). The decrease in WAP and the absence of α-lactalbumin expression suggests that terminal differentiation in *ErbB4ΔIC*-expressing lobuloalveolar epithelium has been disrupted. Similar in situ hybridization analysis performed on mammary glands from female mice at 1 day post-partum yielded equivalent levels of expression of these genes in transgenic and non-transgenic sisters (data not shown).

Stat5 localized to the nucleus of ErbB4ΔIC-expressing mammary epithelium is not phosphorylated at Y694 The condensed lobuloalveoli and pattern of impaired milk-gene expression observed in ErbB4ΔIC-expressing mammary tissue resembles mammary defects observed in mice with *Stat5* gene disruptions (Liu, Robinson et al. 1996; Teglund, McKay et al. 1998). Stat5 expression was determined by immunohistochemistry in sections of mammary glands at 12 days post-partum containing both normal (Fig. 3A) and ErbB4ΔIC-expressing lobuloalveoli (Fig. 3D). Strong immunostaining was detected in the nuclei of both

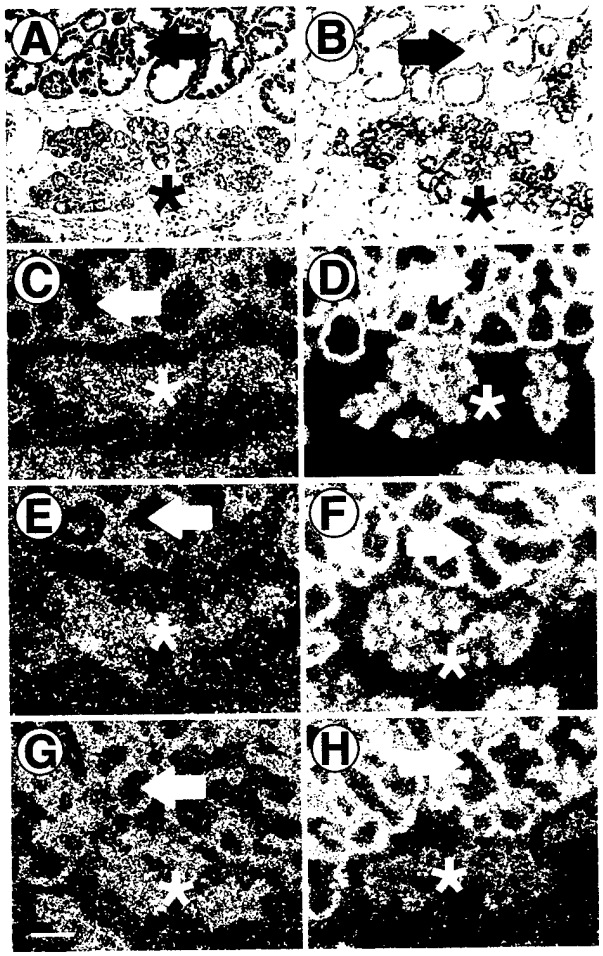


Figure 2. In situ hybridization analysis of milk protein gene expression in ErbB4ΔIC-expressing mammary glands at 12 days post-partum. Sequential sections from a 12 day post-partum mammary gland from an ErbB4ΔIC-expressing mouse were stained with hematoxylin/eosin (A) or stained by immunohistochemistry with anti-Flag antibody (B). Additional sequential sections were analyzed by in situ hybridization with β-casein sense or antisense riboprobes (C and D, respectively), WAP sense or antisense riboprobes (E and F, respectively), or α-lactalbumin sense or antisense riboprobes (G and H, respectively). Expanded secretory lobuloalveoli are indicated by arrows and condensed atypical lobuloalveoli are indicated by asterisks. Bar in G = 100 μm.

normal (compare Fig. 3B and C) and ErbB4ΔIC-expressing lobuloalveoli (compare Fig. 3E and F).

Since functional Stat5 is phosphorylated at Y694 (Reviewed in: (Groner and Gouilleux 1995), I used an antibody specific for Stat5 phosphorylated at Y694 to evaluate the phosphorylation state of Stat5 (Fig. 4). Strong nuclear staining and moderate cytoplasmic staining of phosphorylated Stat5 was detected within normal lobuloalveolar epithelium at 12 days post-partum (Fig. 4C). Immunoreactivity was blocked by preadsorption with the peptide immunogen (Fig. 4B) and was undetectable in

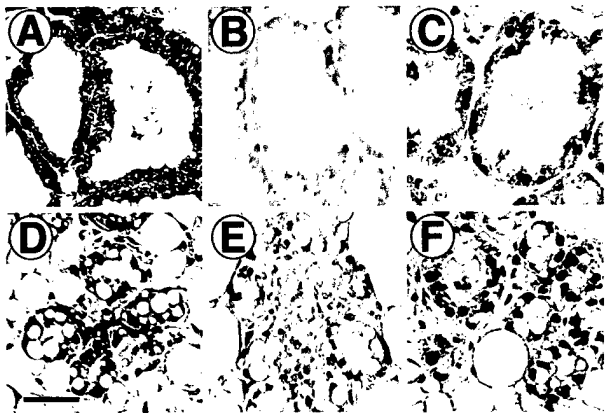


Figure 3. Immunohistochemical detection of Stat5 protein in ErbB4ΔIC-expressing mammary glands at 12 days post-partum. High magnification photomicrographs of expanded lobuloalveoli lacking detectable ErbB4ΔIC expression (A-C) or a different region of the same section containing condensed lobuloalveoli expressing high levels of ErbB4ΔIC protein (D-F). Sections were stained with hematoxylin/eosin (A and D), or stained by immunohistochemistry with rabbit serum negative control (B and E), or rabbit anti-Stat5 (C and F). Bar in D = 30 μm.

sections incubated with affinity purified rabbit IgG control primary antibody (data not shown). Immunoreactive Stat5 and Phospho-Stat5 were detected in both normal mammary glands and phenotypically normal areas of transgenic mammary glands at 1, 3, 6, 9, and 15 days post-partum, but not at day 18 (data not shown). However, at day 12 post-partum, Stat5 was localized to the nucleus (see Fig. 3F), but not phosphorylated in areas expressing ErbB4ΔIC (Fig. 4F). The lack of Y694 phosphorylation of nuclear Stat5 in ErbB4ΔIC-expressing lobuloalveolar epithelium suggests that it is functionally inactive.

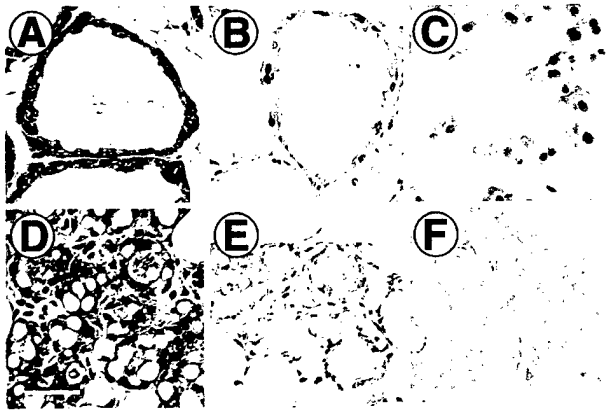


Figure 4. Immunohistochemical detection of Stat5 phosphorylated at Y694 in ErbB4ΔIC-expressing mammary glands at 12 days post-partum. High magnification representations of expanded lobuloalveoli lacking detectable ErbB4ΔIC expression (A-C) or a different region of the same section containing condensed lobuloalveoli expressing high levels of ErbB4ΔIC protein (D-F). Sections were stained with hematoxylin/eosin (A and D), or stained by immunohistochemistry with anti-phosphoStat5 antibody preadsorbed with peptide immunogen and counterstained with methyl green (B and E), or phosphoStat5 antibody without counterstain (C and F). Bar in D = 30 μm.

ErbB4 and Stat5 interaction and ErbB4 mediated phosphorylation of Stat5 at Y694 requires a functional Stat5 SH2 domain Since expression of ErbB4ΔIC appears to inhibit phosphorylation of Stat5 at the regulatory site Y694, it is possible that ErbB4 normally regulates this effector protein during mammary development. To determine if ErbB4 can induce phosphorylation of Stat5a at this site, the proteins were ectopically expressed at high levels in human embryonic kidney 293T cells (Fig. 5). Despite high levels of Stat5a expression in transfected cell lysates (Fig. 5H, lanes 9 and 10, open circle), significant Stat5a tyrosine phosphorylation was only observed when Stat5a was co-expressed with ErbB4 (Fig. 5E, lane 10, open circle). This phosphorylation included Y694, since it was detected by the anti-Stat5 phospho-Y694 antibody (Fig. 5G, lane 10). When Stat5a and ErbB4 were co-expressed in 293T cells, they could be cross-co-immunoprecipitated (Fig. 5D, lane 4; Fig. 5E and 5F, lane 10). Stat5a co-immunoprecipitated with anti-ErbB4 (Fig. 5D, lane 4) was not phosphorylated at Y694 (Fig. 5C, lane 4), suggesting that phosphorylation of Stat5a results in rapid release of Stat5a from an ErbB4/Stat5a complex.

To determine the specificity of ErbB4/Stat5a interaction and ErbB4 mediated phosphorylation of Stat5a on Y694, 293T cells were transfected with mutant *STAT5*a alleles, with inactivating mutations in the SH2 domain (R618 to V) or at Y694 (Y to F). The two Stat5a mutants were expressed at levels comparable to wild-type Stat5a (Fig. 5H, compare lanes 9 and 10 to 11 and 12), but the Stat5a mutants were not phosphorylated at Y694 when co-expressed with ErbB4 (Fig. 6G, lanes 11 and 12). Interestingly, the Stat5a Y694F mutant was tyrosine phosphorylated at sites other than Y694 when co-expressed with ErbB4 (Fig. 6E, lane 12). When expressed alone, the Stat5a mutants Y694F and SH2 were not immunoprecipitated by ErbB4 specific antiserum (data not shown). In summary, ErbB4 and Stat5a were co-immunoprecipitated when co-expressed (Fig. 6D, lane 6 and Fig 6E, closed circle lane 12, and F, lane 12, respectively) and the Stat5a SH2 domain mutation prevented association of ErbB4 and Stat5a (Fig 6D lane 5, E and F lane 11). Hence the interaction between activated ErbB4 and Stat5a and subsequent tyrosine phosphorylation of Stat5a at Y694 requires a functional Stat5a SH2 domain.

Summary of results To elucidate the function of ErbB4 during mouse mammary gland development, I inactivated ErbB4 signaling in the developing mouse mammary gland through the directed expression of dominant negative *ErbB4* as a

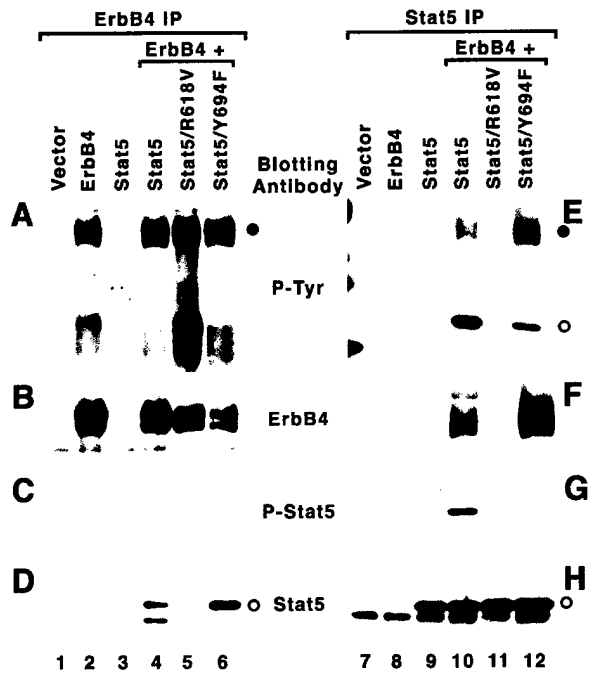


Figure 5. Co-expression of ErbB4 and Stat5a. 293T cells were transfected with various combinations of ErbB4 and Stat5 expression vectors and lysates of transfected cells were prepared 48 hrs post-transfection as described in Materials and Methods. Immunoprecipitations were performed using ErbB4 specific (A-D) or Stat5 specific (E-H) antibodies. Immunoprecipitates were resolved by SDS-PAGE and the 12% acrylamide resolving gel was transferred to nitrocellulose. Western blot analysis was performed using antibodies to detect tyrosine phosphorylated proteins (A and E), ErbB4 (B and F), phosphoStat5 (C and G), or Stat5 (D and H). Stat5 mutants were Stat5/R618V a point mutation which ablates SH2 function, and Stat5/Y694F which eliminates the regulatory Y694. Transfections were with empty vectors (lanes 1 and 7), ErbB4 alone (lanes 2 and 8), Stat5a alone (lanes 3 and 9), ErbB4 + Stat5a (lanes 4 and 10), ErbB4 + Stat5/R618V (lanes 5 and 11), ErbB4 + Stat5/Y694F (lanes 6 and 12). Closed circles in A and E indicate the position of ErbB4 at ca. 190 kDa. Open circles in D, E, and H indicate the position of Stat5a at ca. 95 kDa.

transgene. Despite significant levels of transgene expression throughout mammary gland development an ErbB4ΔIC-specific phenotype was not observed until mid-lactation, at 12 days post-partum. Lobuloalveoli expressing ErbB4ΔIC at 12 days post-partum were condensed, with lumens predominantly filled with secretory lipids, a phenotype resembling normal tissue at late pregnancy. Furthermore, the ErbB4ΔIC-expressing lobuloalveoli failed to terminally differentiate, as evidenced by a lack of α-lactalbumin expression. ErbB4ΔIC also inhibited Stat5 phosphorylation at Y694, suggesting that Stat5 is an important downstream mediator of ErbB4 signaling during lactation.

Corroborative evidence supporting a role for ErbB4 signaling during mid-lactation comes from the timing of ErbB4 activation during mouse mammary gland development, since ErbB4 tyrosine phosphorylation is dramatically enhanced at 14 days post-partum (Schroeder and Lee 1998). These results support the conclusion that ErbB4 signaling plays an important role in lobuloalveolar maintenance and lactation during mid-lactation.

The ErbB4ΔIC-expressing mammary epithelium resembles the phenotype observed in mice with a disrupted *Stat5a* gene (Liu et al., 1996; Teglund et al., 1998). ErbB4 signaling during mid-lactation is required for Stat5 activation, since Stat5 expressed in ErbB4ΔIC-expressing lobuloalveoli was not phosphorylated on the regulatory Y694. Phosphorylation of this residue is essential for some Stat5 functions including dimerization and DNA binding (Groner and Gouilleux, 1995). To our knowledge, this is the first *in vivo* evidence that an EGFR-family member can mediate activation of Stat5.

In transient transfection assays, ErbB4 induced phosphorylation of Stat5a on Y694, and the two proteins could be co-immunoprecipitated in a Stat5 SH2-dependent manner. This suggests that Stat5 is a direct substrate for ErbB4, although I cannot rule out the possible recruitment of a second tyrosine kinase into the complex. Indeed, c-src is an important mediator of Stat5a activation by ErbB family members, and Janus kinases (JAKs) can associate stably with these receptors (Olayioye, Beuvink et al. 1999). The Stat5 consensus docking site (YZXZ, where Z represents a hydrophobic residue) (May, Gerhartz et al. 1996), is present at three sites within the carboxyl-terminus of ErbB4, raising the possibility of a direct interaction between Stat5 and ErbB4.

In contrast to expression of the other EGFR-family members, expression of ErbB4 in breast cancer is associated with favorable prognosis (Bacus, Zelnick et al. 1994; Bacus, Chin et al. 1996; Knowlden, Gee et al. 1998) and a differentiating tumor phenotype (Srinivasan, Poulsom et al. 1998). In this communication, I present *in vivo* evidence demonstrating a role for ErbB4 signaling in terminal differentiation of mammary epithelial cells. My results raise the intriguing possibility that ErbB4 activity in breast cancer cells may activate differentiation pathways and thus antagonize the oncogenic properties of other co-expressed EGFR-family members, including ErbB2. This model is being tested in my laboratory.

Concordance with Statement of Work

The following is a descriptive summary of the work completed to date and its relation to the proposed Statement of Work. Explanations for aims or tasks not completed are provided where appropriate.

Technical Objective 1. Developmental expression of receptors and growth factors.

Task 1. Quantitative RT-PCR of growth factors from normal and ErbB2 overexposing staged mouse mammary tissue.
Status: This work has been completed by another laboratory and published (Schroeder and Lee 1998).

Task 2. Perform quantitative RT-PCR analysis in hormonally manipulated mice.
Status: This work will be pursued by Schroeder and Lee (Schroeder and Lee 1998).

Task 3. *In situ* hybridization analysis of receptors and growth factors identified in mouse tissue during Task 1.
Status: These experiments have been completed by Dr. David Lee at the University of North Carolina.

Technical Objective 2. Response of mammary epithelium to growth factor implants.

Task 4. NRGα and NRGβ implants - complete work in progress.
Status: Work completed.

Task 5. Analysis of implants containing growth factors identified in Task 1 in normal mice.
Status: These experiments require milligram amounts of growth factor. Unfortunately I could not find a source for the growth factors under study.

Task 6. Analysis of growth factors in hormonally manipulated mice.
Status: These experiments require milligram amounts of growth factor. Unfortunately I could not find a source for the growth factors under study.

Task 7. Analysis of growth factors in ErbB2 overexposing mice during tumor-free latency period.
Status: These experiments require milligram amounts of growth factor. Unfortunately I could not find a source for the growth factors under study.

Technical Objective 3. Alter expression of receptors and growth factors to determine role in regulation of ErbB2 signaling and breast cancer.

Task 8. Place long-term implants (3-6 months) into ErbB2 overexposing mice. Pellets will contain possible ErbB2 regulating growth factors identified in Task 7. Analyze effects on ErbB2 signaling and tumor development.
Status: These experiments require milligram amounts of growth factor. Unfortunately I could not find a source for the growth factors under study.

Task 9. Construct and subclone dominant negative receptor mutants with MCMV IE promoter into retroviral vector. Receptor selection based upon results from Aim 1.
Status: An alternative strategy has been developed. See status of Task 11.

Task 10. Analysis of dominant negative mutants for activity in cell lines which express receptors.
Status: An alternative strategy has been developed. See status of Task 11.

Task 11. Express dominant negative mutants in mammary epithelium of ErbB2 overexposing mice and analyze effects on ErbB2 signaling and tumor development.
Status: An alternative strategy has been used to identify the roles of ErbB2 and its signaling partners in normal and malignant mammary development. Transgenic mice expressing dominant negative ErbB2 has been published and mice expressing dominant negative ErbB4 are described in this report. A similar strategy has been used to analyze EGFR signaling (Xie, Paterson et al. 1997) and I will examine ErbB3 signaling using transgenic expression of dominant negative receptors.

References

Bacus, S. S., D. Chin, et al. (1996). "Type 1 receptor tyrosine kinases are differentially phosphorylated in mammary carcinoma and differentially associated with steroid receptors." Am. J. Pathol. **148**: 549-558.

Bacus, S. S., C. R. Zelnick, et al. (1994). "Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers: implication for tumor biology and clinical behavior." Am. J. Clin. Pathol. **102**: S13-S24.

Groner, B. and F. Gouilleux (1995). "Prolactin-mediated gene activation in mammary epithelial cells." Curr. Opin. Genet. Dev. **5**: 587-594.

Hynes, N. E. and D. F. Stern (1994). "The biology of *erbB-2/neu/HER-2* and its role in cancer." Biochim. Biophys. Acta **1198**: 165-184.

Jones, F. E., D. J. Jerry, et al. (1996). "Heregulin induces *in vivo* proliferation and differentiation of mammary epithelium into secretory lobuloalveoli." Cell Growth Differ. **7**: 1031-1038.

Jones, F. E. and D. F. Stern (1999). "Expression of dominant-negative ErbB2 in the mammary gland of transgenic mice reveals a role in lobuloalveolar development and lactation." Oncogene **18**: 3481-3490.

Knowlden, J. M., J. M. W. Gee, et al. (1998). "c-*erb*B3 and c-*erb*B4 expression is a feature of the

endocrine responsive phenotype in clinical breast cancer." Oncogene **17**: 1949-1957.

Liu, X., G. W. Robinson, et al. (1996). "Stat5a is mandatory for adult mammary gland development and lactogenesis." Genes Dev. **11**: 179-186.

May, P., C. Gerhartz, et al. (1996). "Comparative study on the phosphotyrosine motifs of different cytokine receptors involved in STAT5 activation." FEBS Lett. **394**: 221-226.

Nass, S. J., H. A. Hahm, et al. (1998). "Breast cancer biology blossoms in the clinic." Nature Med. **4**: 761-762.

Olayioye, M. A., I. Beuvink, et al. (1999). "ErbB receptor-induced activation of Stat transcription factors is mediated by Src tyrosine kinases." J. Biol. Chem. **274**: 17209-17218.

Schroeder, J. A. and D. C. Lee (1998). "Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland." Cell Growth Differ. **9**: 451-464.

Srinivasan, R., R. Poulsom, et al. (1998). "Expression of the c-erbB-4/HER4 protein and mRNA in normal human fetal and adult tissues and in a survey of nine solid tumour types." J. Pathol. **185**: 236-245.

Teglund, S., C. McKay, et al. (1998). "Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses." Cell **93**: 841-850.

Xie, W., A. J. Paterson, et al. (1997). "Targeted expression of a dominant negative epidermal growth factor receptor in the mammary gland of transgenic mice inhibits pubertal mammary duct development." Mol. Endocrinol. **11**: 1766-1781.

Appendix

Key Research Accomplishments

- Identified the growth factor, heregulin1, as a differentiation factor in the mammary gland (Jones, Jerry et al. 1996).
- Determined the normal function of ErbB2 during breast development (Jones and Stern 1999).
- Determined the normal function of ErbB4 during breast development.
- Identified Stat5 as an important mediator of ErbB4 signaling during mammary differentiation.

Reportable Outcomes

Manuscripts

Jones, F. E. and D. F. Stern. 1999. Expression of dominant-negative ErbB2/Neu in the mammary gland of transgenic mice reveals a role in lobuloalveolar development and lactation. Oncogene **17**:3481-3490.

Jones, F. E., D. J. Jerry, B. C. Guarino, G. C. Andrews, D. F. Stern. 1996. Heregulin induces *in vivo* proliferation and differentiation of mammary epithelium into secretory lobuloalveoli. Cell Growth and Differentiation **7**:1031-1038.

Meeting Abstracts

Frank E. Jones and David F. Stern. ErbB4 signaling is required for activation of Stat5 in the lactating breast.
Specificity in Signal Transduction. Keystone, CO. April 9-14, 1999.

Frank E. Jones and David F. Stern. Dominant-negative erbB-2/neu receptor impairs mammary gland development.
The 9th Annual Growth Factor and Signal Transduction Conference. Iowa State University. September 25-28, 1997.

Frank E. Jones, D. Joseph Jerry, and David F. Stern. Expression of epidermal growth factor receptor family members and their ligands in the developing mouse mammary gland.
Basic and Clinical Aspects of Breast Cancer. Keystone, CO. March 7-12, 1997.

Frank E. Jones, D. Joseph Jerry, Brad C. Guarino, Glenn C. Andrews, and David F. Stern. Heregulin/NDF implants induce epithelial differentiation and milk-production within mammary glands of virgin female mice.
Tyrosine Phosphorylation and Cell Signaling. The Salk Institute. August 21-25, 1996

Employment

Research Scientist at the University of Scranton Institute of Molecular Biology and Medicine.
Start date: July 19

Oncogene (1999) 18, 3481–3490

http://www.stockton-press.co.uk/onc

Expression of dominant-negative ErbB2 in the mammary gland of transgenic mice reveals a role in lobuloalveolar development and lactation

Frank E Jones[1] and David F Stern*,[1]

[1]*Department of Pathology, BML-342, Yale University School of Medicine, P.O. Box 208023, New Haven, Connecticut 06520-8023, USA*

Overexpression of the receptor tyrosine kinase ErbB2/HER2/Neu (ErbB2) occurs in 15–40% of human breast cancers. To determine the function of ErbB2 signaling during normal mouse mammary gland development, we expressed a carboxyl-terminal truncated dominant negative allele of *ErbB2* (ErbB2ΔIC) in the developing mouse mammary gland. Despite ErbB2ΔIC expression within mammary glands of pubescent virgin and pregnant mice, a phenotype was not observed until late in pregnancy. At 1 day *post-partum*, lactationally active, distended lobuloalveoli failed to form. This phenotype was exaggerated in multiparous females expressing ErbB2ΔIC. Immunohistochemical staining for ErbB2-ΔIC revealed a concordance between high levels of ErbB2ΔIC protein expression and the absence of lactational products within the lumens of ErbB2ΔIC stained lobuloalveoli. These results demonstrate that ErbB2 signaling is required for proper mammary development and lactation at parturition.

Keywords: ErbB2; dominant negative receptor mutant; transgenic mice; mammary gland development

Introduction

Breast cancer is the most commonly diagnosed cancer and the second most frequent cause of cancer mortality in North American women (Parker *et al.*, 1996). Normal and malignant mammary epithelial growth is regulated by the activities of circulating hormones including estrogen, progesterone, and prolactin (Topper and Freeman, 1980; Vonderhaar, 1984) and locally produced growth factors (DiAugustine *et al.*, 1997; Dickson and Lippman, 1995). Perturbation of signaling cascades regulated by these mammary growth modulators can lead to malignant transformation of the breast.

One important mediator of growth factor signaling, the receptor tyrosine kinase ErbB2/HER2/Neu (referred to here as ErbB2) is amplified and overexpressed in 15–40% of human breast carcinomas (Hynes and Stern, 1994). Overexpression of ErbB2 is associated with poor prognosis, especially in patients with lymph node involvement (Hynes and Stern, 1994; Revillion *et al.*, 1998; Slamon *et al.*, 1987). In phase III clinical trials of a recombinant humanized anti-ErbB2 monoclonal antibody, targeting of breast cancer cells overexpressing ErbB2 results in growth inhibition and regression of tumors (reviewed in: Nass *et al.*, 1998). These results demonstrate that ErbB2 will be a valuable target for rational therapeutic treatment of breast cancer patients with tumors overexpressing ErbB2. Despite the clinical importance of ErbB2 in breast cancer, the exact function of ErbB2 signaling during normal mammary gland development has not been established.

ErbB2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (Coussens *et al.*, 1985). Additional members of this family are the EGFR itself, ErbB3 (Kraus *et al.*, 1989) and ErbB4 (Plowman *et al.*, 1993). There are at least nine different genes that encode growth factors belonging to the epidermal growth factor (EGF)-family. Each of these factors has a different intrinsic ability to bind to and activate ErbB-family receptors (reviewed in: Riese II and Stern, 1998; Salomon *et al.*, 1995). A ligand that binds to and activates ErbB2 has not been identified. ErbB2 signaling can be activated, however, through heterodimer formation with other ligand-bound ErbB-family members. For example, the EGFR ligand, EGF does not bind to ErbB2 but does activate ErbB2 when ErbB2 is coexpressed with EGFR (Stern *et al.*, 1986; Stern and Kamps, 1988). This is mediated by induction of EGFR/ErbB2 heterodimers (Spivak-Kroizman *et al.*, 1992; Wada *et al.*, 1990). Although each ligand-activated EGFR-family member can form a signaling dimer with most other members of this receptor family, ErbB2 is the preferred heterodimer partner (Graus-Porta *et al.*, 1997; Tzahar *et al.*, 1996). Moreover, ligand stimulated receptors compete for dimer formation with ErbB2 (Chen *et al.*, 1996; Karunagaran *et al.*, 1996). The distinct signaling activities of each receptor/ligand combination results in diverse cellular responses mediated through heterodimer formation with ErbB2 (DiFiore *et al.*, 1990; Kokai *et al.*, 1989; Riese II *et al.*, 1998). Collectively, these results support a signaling paradigm in which ErbB2 fulfills a role as central mediator of EGFR family signal transduction.

Expression of ErbB2, its receptor signaling partners, and several ErbB2 agonists has been detected within the developing rodent mammary gland. ErbB2 is expressed within stromal and epithelial cells of virgin mouse mammary glands and lobuloalveolar epithelium from pregnant and lactating mice (DiAugustine *et al.*, 1997; Sebastian *et al.*, 1998; Schroeder and Lee, 1998). Similar cellular and temporal expression patterns have been documented for EGFR (DiAugustine *et al.*, 1997; Ederly *et al.*, 1985; Sebastian *et al.*, 1998; Schroeder and Lee, 1998), ErbB3 (Sebastian *et al.*, 1998; Schroeder and Lee, 1998; Yang *et al.*, 1995), and ErbB4 (Sebastian *et al.*, 1998; Yang *et al.*, 1995),

*Correspondence: DF Stern
Received 15 September 1998; revised 14 January 1999; accepted 14 January 1999

although the expression of ErbB4 is highest during pregnancy (Schroeder and Lee, 1998). Likewise, the EGFR agonists, EGF (Schroeder and Lee, 1998; Snedeker *et al.*, 1991), TGFα (Schroeder and Lee, 1998; Snedeker *et al.*, 1991), and AR (Kenney *et al.*, 1995; Schroeder and Lee, 1998) are expressed in both virgin and pregnant mammary tissue. In contrast to these ligands, significant expression levels of the ErbB3 and ErbB4 ligand, NRG1, are only detected during pregnancy (Yang *et al.*, 1995). The variable expression of individual EGFR family members and ligands suggests that this receptor family has multiple functions during mammary gland development.

Substantial evidence supports a role for the EGFR and its ligands in mammary ductal proliferation and morphogenesis at puberty. Mammary implants containing EGF (Coleman *et al.*, 1988; Snedeker *et al.*, 1991), TGFα (Snedeker *et al.*, 1991), or AR (Kenney *et al.*, 1996) induce ductal morphogenesis and proliferating terminal end bud (TEB) formation in ovariectomized mice. Consistent with the proliferative effects of EGFR ligands on mammary development, inhibition of EGFR signaling with a dominant negative EGFR results in decreased ductal proliferation and impaired morphogenesis in the virgin mammary gland (Xie *et al.*, 1997).

In contrast to ligands for the EGFR, signaling induced by the ErbB3 and ErbB4 ligand, NRG1, is associated with mammary differentiation. In the AU565 human breast cancer cell line, NRG1 induces a differentiation phenotype (Bacus *et al.*, 1992; Peles *et al.*, 1992) which requires ErbB2 expression (Yoo and Hamburger, 1998). Moreover, activated ErbB2 is capable of forming alveolar-like structures in mammary epithelial cells identical to those observed following NRG1 treatment (Niemann *et al.*, 1998). *In vivo*, lobuloalveoli induced by mammary implants containing NRG1, but not TGFα, produce secretory products including the milk-protein β-casein (Jones *et al.*, 1996). Taken together with the weak expression of NRG1 in nulliparous mice, these results suggest that NRG1 induces distinct biological responses from TGFα and that expression of NRG1 during pregnancy is coupled to ErbB2 signaling during mammary epithelial differentiation.

The ability of ErbB2 to be activated through dimerization with all other EGFR-family members suggests that ErbB2 plays a central role in both the proliferative phase of mammary development at puberty, and mammary differentiation during pregnancy and lactation. To elucidate the mechanisms that govern ErbB2 mediated breast development, we inactivated signaling by endogenous ErbB2 during mouse mammary gland development through transgenic expression of a mutant ErbB2 with dominant negative activity. The results demonstrate that ErbB2 signaling is required for the terminal stages of lobuloalveolar development and lactation at parturition.

Results

Expression and dominant negative activity of a truncated ErbB2

Overexpression of truncated ErbB2 lacking the entire kinase domain and C-terminal tyrosine phosphorylation sites inhibits normal ErbB2 signaling *in vitro* (Qian *et al.*, 1994) and can severely compromise ErbB2-mediated tumor formation in the nude mouse (Qian *et al.*, 1996). We constructed a similar dominant negative mutant by replacing sequences encoding the rat ErbB2 endodomain, downstream of A694, with sequences encoding an influenza virus hemagglutinin (HA) epitope-tag. These coding sequences were placed under control of the mouse mammary tumor virus (MMTV) promoter to direct transgene expression within mouse mammary epithelial cells (Figure 1a). The resulting construct,

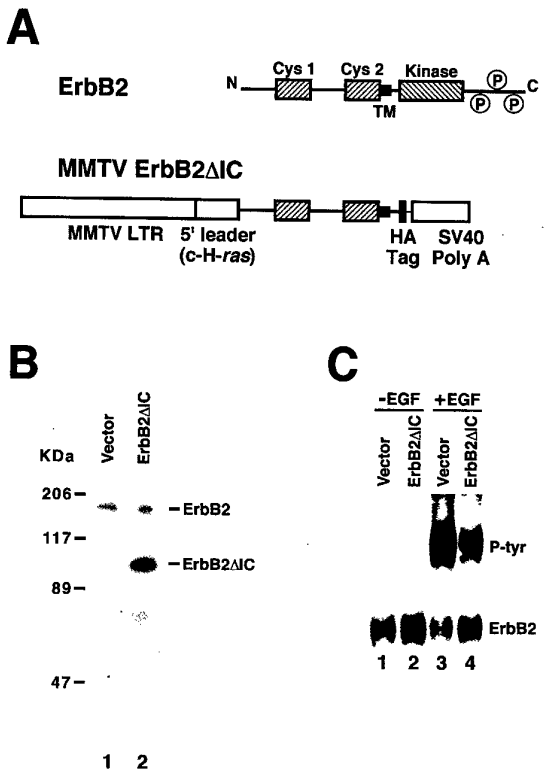


Figure 1 (**a**) Schematic representation of ErbB2 and the transgene MMTV-ErbB2ΔIC. Locations of cysteine-rich domains (Cys1 and Cys2), transmembrane region (TM), tyrosine kinase domain (Kinase), and carboxyl-terminal phosphotyrosines (P) are indicated. The ErbB2ΔIC transgene is downstream of the mouse mammary tumor virus (MMTV) LTR and 5′ leader sequence from c-H-*ras*. ErbB2ΔIC encodes the entire ErbB2 extracellular domain, the transmembrane domain, followed by the juxtamembrane region to residue 694, HA epitope-tag (HA Tag), followed by polyadenylation and splice signals from SV40 (SV40 Poly A). (**b**) Expression of endogenous ErbB2 and ErbB2ΔIC. FR3T3 cell clones transfected with empty vector (lane 1) or MMTV-ErbB2ΔIC (lane 2) were metabolically labeled with ^{35}S-methionine/cysteine and grown in the presence of 1 μM dexamethasone to activate the MMTV promoter. Immunoprecipitations were performed using an antibody directed against the ectodomain of rat ErbB2. Molecular weight markers are indicated to the left and the positions of endogenous ErbB2 and ErbB2ΔIC are indicated. (**c**) Dominant negative activity of MMTV-ErbB2ΔIC. FR3T3 cell clones transfected with empty vector (lanes 1 and 3) or MMTV-ErbB2ΔIC (lanes 2 and 4) were cultured with 1 μM dexamethasone for 24 h and then mock stimulated (lanes 1 and 2) or stimulated with EGF (lanes 3 and 4) for 10 min at room temperature. ErbB2 was immunoprecitated from cell lysates and probed for phosphotyrosine by Western blot analysis (upper panel). The blot was then stripped and reprobed for ErbB2 (lower panel). The positions of phosphorylated endogenous ErbB2 (P-tyr) and total endogenous ErbB2 (ErbB2) are indicated

designated MMTV-ErbB2ΔIC, encodes a protein of 704 residues with a predicted molecular mass of 105 KDa. The ErbB2ΔIC protein was expressed in FR3T3 rat embryonic fibroblast cells transfected with MMTV-ErbB2ΔIC (Figure 1b, lane 2) but not cells transfected with an empty vector (Figure 1b, lane 1). ErbB2 is phosphorylated in response to EGF when coexpressed with EGFR (Stern *et al.*, 1986; Stern and Kamps, 1988). To determine the effects of ErbB2ΔIC expression on the transphosphorylation of ErbB2 in response to EGF, FR3T3 cell lines expressing ErbB2ΔIC or the MMTV vector alone were mock treated or treated with EGF. Endogenous ErbB2 was immunoprecipitated from treated cells and the levels of receptor tyrosine phosphorylation was determined by anti-phosphotyrosine Western blot analysis. ErbB2 tyrosine phosphorylation was not observed in mock treated cell lines (Figure 1c, lanes 1 and 2). In contrast, EGF treatment resulted in high levels of ErbB2 tyrosine phosphorylation in a cell line containing the empty vector (Figure 1c, lane 3). Tyrosine phosphorylation of ErbB2 was inhibited, however, by expression of ErbB2ΔIC (Figure 1c, lane 4). Immunoprecipitation of ErbB2 from each cell treatment was confirmed by stripping and reprobing the blot for ErbB2 (Figure 1c, bottom panel). These results demonstrate that the ErbB2 truncation mutant, ErbB2ΔIC, has dominant negative activity when coexpressed with wild-type ErbB2.

Transgenic mice derived from MMTV-ErbB2ΔIC

To determine the effect of dominant negative ErbB2 activity within the developing mammary gland, transgenic mice were derived by injecting MMTV-ErbB2ΔIC DNA into the pronuclei of fertilized one-cell zygotes from B6SJL/F2 mice. Four founders with transgene integration (determined by polymerase chain reaction; PCR) were crossed into a FVB strain and transgene expression by the F2 female offspring was determined by Northern blot analysis using an ErbB2ΔIC-specific probe. Transgene expression was detected in mid-pregnant mammary glands from offspring of the two founder transgene lines designated 5286 and 5289 (data not shown). Phenotypic analysis of mammary glands from mice expressing ErbB2ΔIC was performed on F3 females derived by crossing founder line 5289 F2 mice with FVB strain mice. The phenotype of line 5289 was confirmed by analysis of a second founder line, 5286.

Expression of the ErbB2ΔIC transgene in the mammary gland

To determine the temporal expression pattern of ErbB2ΔIC, RNA was isolated from the number 4 inguinal mammary gland of developmentally staged female mice and subjected to analysis by ribonuclease protection assay (RNPA). The 320 bp ErbB2ΔIC riboprobe hybridizes to the extreme 3′ end of the ErbB2ΔIC transgene, including unique sequences corresponding to the HA-tag, resulting in a protected fragment of 240 bp (Figure 2, lanes 1 and 4, respectively). A similar protected fragment was never detected in mammary glands from developmentally staged non-transgenic siblings (e.g. Figure 2, lane 5, 1 day *post-partum*). A protected fragment of 204 bp representing endogenous ErbB2 RNA was detected in empty vector-transfected cells (lane 3). Transgene

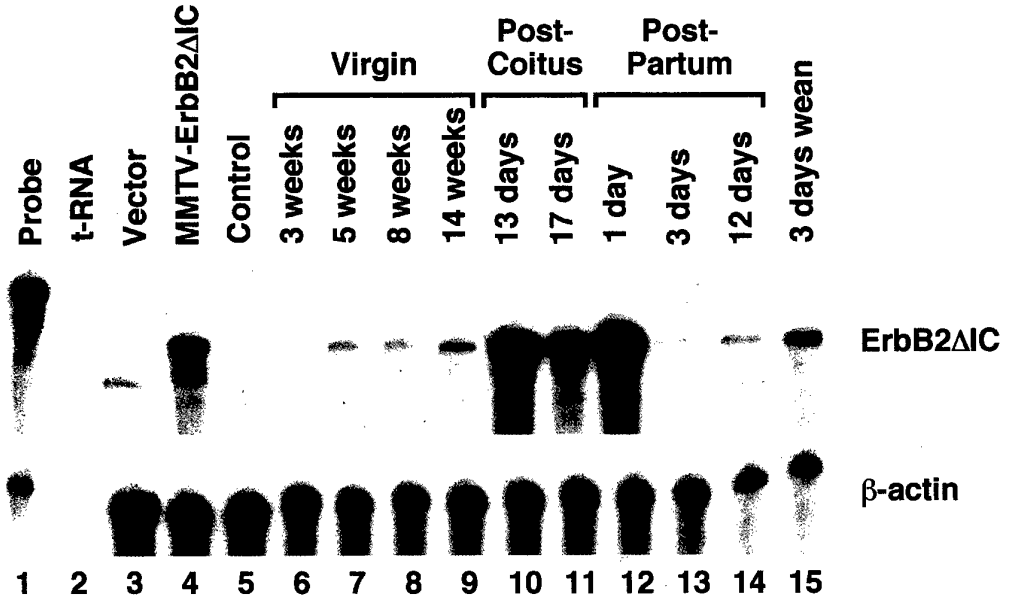


Figure 2 Expression analysis of ErbB2ΔIC RNA in developmentally staged mammary glands. RNA (20 μg) isolated from the number 4 inguinal mammary gland was hybridized with ^{32}P-labeled anti-sense riboprobe corresponding to the carboxyl-terminal 236 bp of ErbB2ΔIC and subjected to RNase protection analysis. Protected fragments corresponding to ErbB2ΔIC and β-actin are indicated. Negative controls included 20 μg each of tRNA, a FR3T3 cell clone transfected with empty vector (Vector), RNA from a non-transgenic sibling at 1 day *post-partum* (Control). RNA (20 μg) from a FR3T3 cell clone transfected with MMTV-ErbB2ΔIC was a positive control. β-actin served as an internal control for RNA loading

expression was first detected in virgin females as they entered puberty at 5 weeks, and expression levels increased slightly with age with maximal expression in the mature nulliparous mammary gland at 14 weeks (Figure 2, lanes 6–9). Expression levels throughout pregnancy were several-fold greater than observed within the mature virgin mammary gland (Figure 2, lanes 10 and 11). (In other experiments, the transgene was expressed at similarly high levels beginning at day 11 *post-coitus*, the earliest time point assayed.) The highest level of expression, however, was observed at 1 day *post-partum* (Figure 2, lane 12). The window of high level *post-partum* transgene expression was restricted, with a decrease in expression observed at both 3 and 12 days *post-partum* (Figure 2, lanes 13 and 14). Mammary gland involution, following 3 days of weaning, resulted in a return of ErbB2ΔIC expression to pre-pregnancy levels (Figure 2, lane 15).

Mammary gland expression of ErbB2ΔIC inhibits lobuloalveolar development at parturition

Mammary gland development can be broadly divided into two hormonally regulated phases: (1) ductal proliferation and branching morphogenesis in the pubescent virgin and (2) lobuloalveolar development with epithelial terminal differentiation during pregnancy and lactation. To determine the effects of ErbB2ΔIC expression on mouse mammary gland development, whole mounts and histologically-stained paraffin-embedded sections of mammary glands from ErbB2ΔIC expressing virgin mice were examined at 3, 5, 7, 10 and 14 weeks of age. Despite the expression of ErbB2ΔIC within the virgin mammary gland after 5 weeks (Figure 2), we did not detect effects on terminal end-bud formation, ductal branching, ductal growth, or cellular morphology within the nulliparous mammary gland (data not shown).

With the onset of pregnancy, proliferation of lateral and terminal ductal epithelial buds initiates ductal side-branching and the accumulation of alveolar units. At 19 days *post-coitus*, some lobuloalveoli were expanded in non-transgenic mice (open arrow, Figure 3a) and the ducts become slightly distended (arrowhead, Figure 3a). High levels of ErbB2ΔIC expression during pregnancy had no effect on ductal side-branching or alveolar development up to and including 17 days *post-coitus* (data not shown). Evidence for inhibition of alveolar growth in ErbB2ΔIC-expressing mice was first observed at 19 days *post-coitus*. Although distended ducts were observed in wholemounts of ErbB2ΔIC expressing mice at this stage (arrowhead, Figure 3b), the lobuloalveoli of these mice remained condensed (filled arrow, Figure 3b).

At 1 day *post-partum*, lobuloalveoli of control mice expand as the alveolar lumens become engorged with lactation products (arrow, Figure 3c). In control mice, the ducts are obscured by the extensive expansion of lobuloalveoli. Likewise, the lobuloalveoli of multiparous control mice at 1 day *post-partum* were distended (arrow, Figure 3e), but to a much greater extent than those of uniparous control mice (compare Figure 3e to Figure 3c). A dramatic phenotype was observed in ErbB2ΔIC-expressing mice at 1 day *post-partum*. Alveoli from both uniparous and multiparous transgenic mice remained condensed (filled arrows, Figure 3d and f). Furthermore, distended ducts were clearly visible in wholemounts from the ErbB2ΔIC transgenic mice at 1 day *post-partum* (arrowheads, Figure 3d and f), suggesting that lobuloalveolar but not ductal epithelial cells are adversely effected by ErbB2ΔIC expression. The ErbB2ΔIC phenotype observed at 1 day *post-partum* was accentuated in multiparous females (>6 pregnancies; compare Figure 3d and 3f), presumably because multiparity results in both higher levels and more uniform transgene expression driven by the MMTV LTR. At 3 days *post-partum*, epithelial proliferation results in further accumulation and expansion of lobuloalveoli in control mice (Figure 3g). Although distended lobuloalveoli were prominent within transgenic mice at 3 days *post-partum*, the lobuloalveoli were still much smaller when compared to control mice (Figure 3h). The recovery of lobuloalveolar development in the ErbB2ΔIC transgenic mice at this stage may be related to the dramatic decrease in transgene expression observed during lactation (Figure 2). The ErbB2ΔIC expressing mammary gland was indistinguishable from non-transgenic controls at 12 days *post-partum* (data not shown), a stage with maximal lactation and low transgene expression.

Histological analysis of mammary glands from control mice at 19 days *post-coitus* revealed expanded lobuloalveoli with the accumulation of cellular and lumenal secretory lipids (arrow, Figure 4a). In some alveoli, lumenal proteinacious material representing the early stages of terminal differentiation and lactation was detected (open arrowhead, Figure 4a). Although secretory lipids were detected within lobuloalveoli of ErbB2ΔIC expressing mice at 19 days *post-coitus* (arrow, Figure 4b), the lobuloalveoli were much smaller than those of control mice. Reduced amounts of proteinacious material were detected in the lumens of lobuloalveoli from ErbB2ΔIC-expressing mice (open arrowhead, Figure 4b).

At 1 day *post-partum*, secretory epithelium lining the lobuloalveoli of control mice is no longer cuboidal, but has a flattened appearance, indicating that the cells have undergone secretory differentiation (Figure 4c). In addition, the lumens of control mice are filled with granular proteinacious material (open arrowhead, Figure 4c) and a small amount of lipid indicating that normal milk production is in progress (Figure 4c). In multiparous control animals, the lobuloalveoli are much larger than uniparous controls and the lumens are empty, presumably due to the efficient secretory process of these glands (Figure 4e). A majority of alveoli from ErbB2ΔIC expressing mice at 1 day *post-partum* retained the cuboidal secretory structure observed at 19 days *post-coitus* (Figure 4b and d and data not shown). Lumenal products present in lobuloalveoli from ErbB2ΔIC expressing mice resemble secretory lipids typically observed during late pregnancy (compare arrows in Figure 4d and f with Figure 4a and b). Multiparity dramatically exaggerated the inhibitory effect of ErbB2ΔIC expression on lobuloalveolar development. Condensed lobuloalveoli with cuboidal epithelium predominated in mammary glands from multiparous transgenic females at 1 day *post-partum* (Figure 4f). At 3 days *post-partum* the alveolar epithelia of both transgenic and control mice are rounded as they enter the first phase of *post-partum*

proliferation (Traurig, 1967a) (Figure 4g and h). Despite the apparent recovery of epithelial cell function, lobuloalveoli of ErbB2ΔIC expressing mice at 3 days *post-partum* remained much smaller than those of non-transgenic controls.

ErbB2ΔIC expression is associated with non-secreting lobuloalveoli

In contrast to the uniform structure of lactating mammary glands from non-transgenic siblings, mammary glands from ErbB2ΔIC-expressing mice displayed a high degree of intragland heterogeneity (Figure 5a). A subset of alveolar clusters with cuboidal epithelial cells always lacked lumenal secretory products (arrows). In contrast, other alveolar clusters with slightly flattened epithelium contained secretory lipids typically observed late in pregnancy (arrowheads; compare to Figure 4a). To determine if this heterogeneity is related to ErbB2ΔIC expression levels, transgene expression was determined by immunohistochemistry (IHC) (Figure 5b).

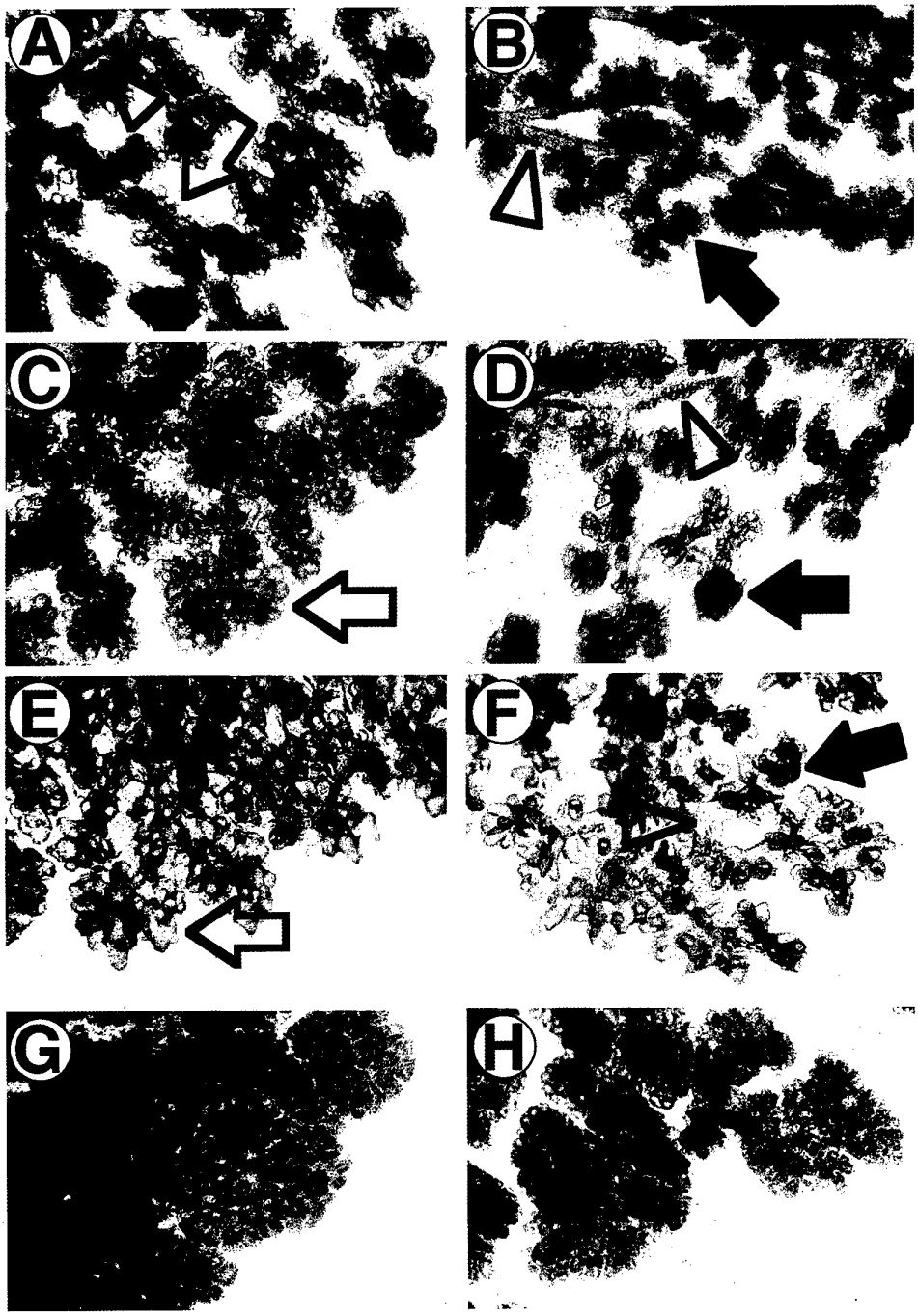


Figure 3 Wholemount analysis of developmentally staged mammary glands from non-transgenic control (**a**, **c**, **e** and **g**) and ErbB2ΔIC transgenic mice (**b**, **d**, **f** and **h**). The entire number 4 inguinal mammary gland at 19 days *post-coitus* (**a** and **b**), 1 day *post-partum* (**c** and **d**), multiparous mice at 1 day *post-partum* (**e** and **f**), or 3 days *post-partum* (**g** and **h**) was prepared as wholemount and stained with carmine. Distended ducts are indicated by arrowheads. Expanded and condensed lobuloalveoli are indicated by open and filled arrows, respectively

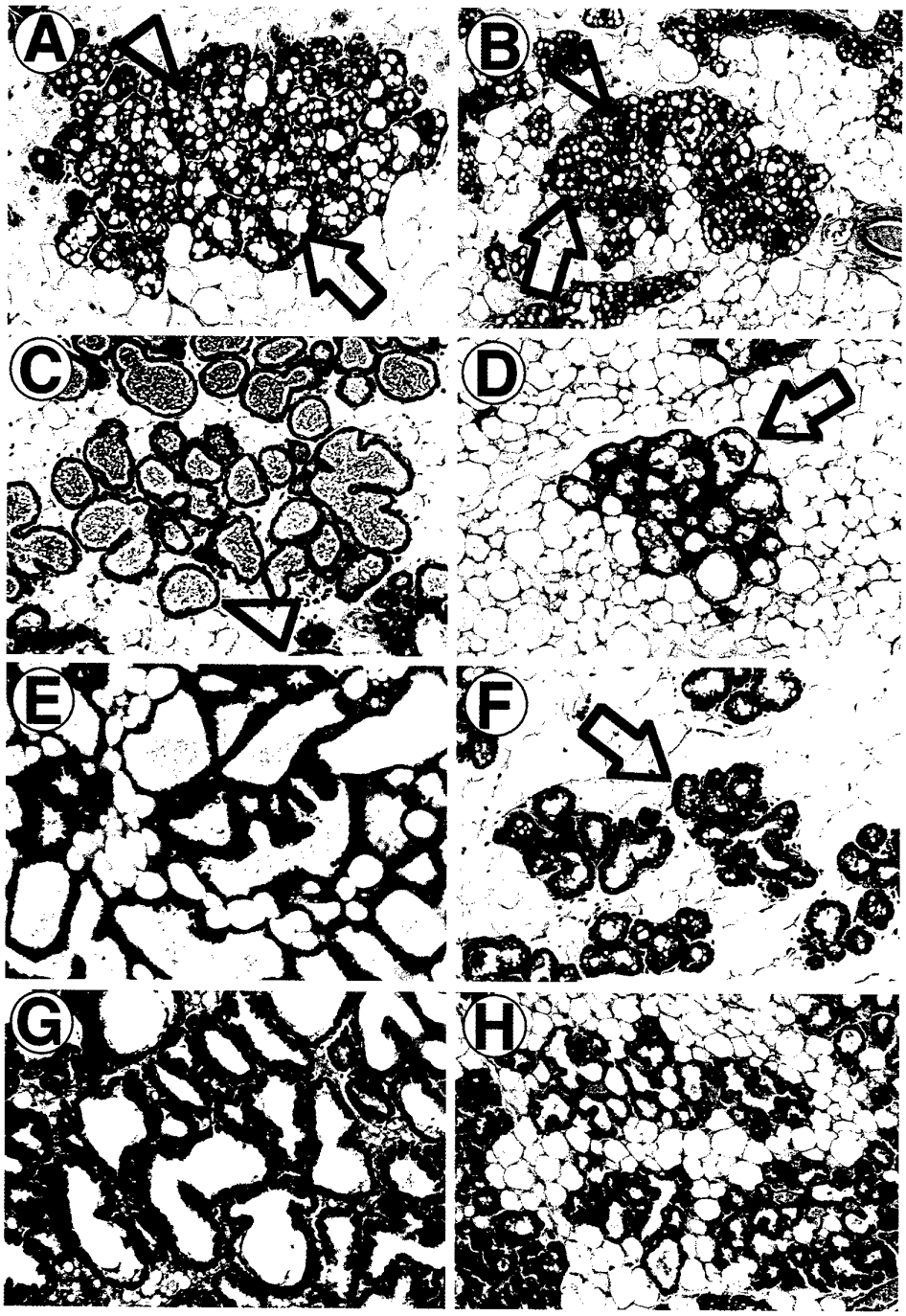


Figure 4 Histological analysis of developmentally staged mammary glands from non-transgenic control (**a**, **c**, **e** and **g**) and ErbB2ΔIC transgenic mice (**b**, **d**, **f**, and **h**). A portion of the number 4 inguinal mammary gland at 19 days *post-coitus* (**a** and **b**), 1 day *post-partum* (**c** and **d**), multiparous mice at 1 day *post-partum* (**e** and **f**), or 3 days *post-partum* (**g** and **h**) was fixed in paraformaldehyde, embedded in paraffin and stained with hematoxylin/eosin. Proteinacious and lipid lumenal secretory products are indicated by open arrowheads and open arrows, respectively

Anti-HA, which recognizes the HA epitope-tagged ErbB2ΔIC, yielded specific staining of epithelium from condensed lobuloalveolar clusters (arrows, Figure 5b). At higher magnifications membrane staining of the same cells was observed (data not shown). The lumens of ErbB2ΔIC-staining lobuloalveolar clusters lacked secretory products (compare areas marked with arrows in Figure 5a and b). ErbB2ΔIC was never detected in epithelium of lobuloalveolar clusters which contained secretory products (arrowheads, Figure 5a and b) or non-transgenic controls (data not shown). These results demonstrate a direct concordance between detectable levels of ErbB2ΔIC expression and the inhibition of lobuloalveolar epithelial differentiation and secretory activity at 1 day *post-partum*.

Despite the dramatic inhibition of lobuloalveolar development and secretory processes observed within mammary glands of ErbB2ΔIC expressing mice at

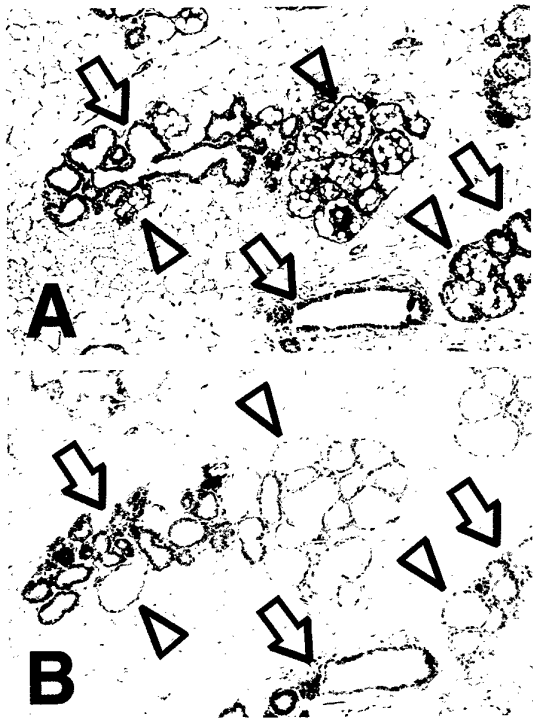


Figure 5 Immunohistochemical localization of ErbB2ΔIC in the mammary gland at 1 day *post-partum*. Sequential sections from a 1 day *post-partum* mammary gland of an ErbB2ΔIC expressing mouse were stained with hematoxylin/eosin (**a**) or analysed by immunohistochemistry using a HA specific primary antibody (**b**) (see Materials and methods). ErbB2ΔIC expressing lobuloalveoli are indicated by arrows, and lobuloalveoli lacking significant ErbB2ΔIC expression are indicated by arrowheads

parturition, milk was clearly visible within the stomachs of pups throughout lactation, and there was no apparent consequence of ErbB2ΔIC expression on nursing pups (data not shown). The incomplete inhibition of terminal differentiation, resulting in functionally lactating ErbB2ΔIC-expressing mammary glands, may be a result of the variegated transgene expression observed at 1 day *post-partum* (Figure 5b).

Discussion

Locally synthesized EGF family hormones and their receptors play important roles in normal and neoplastic mammary development (DiAugustine *et al.*, 1997; Dickson and Lippman, 1995). Indeed, a large body of evidence indicates that the EGFR family member ErbB2 is a key determinant in the ontogenesis and progression of human breast cancer (reviewed in: Hynes and Stern, 1994). In order to inactivate normal ErbB2 signaling during mammary development, we directed expression of a dominant negative *ErbB2* allele (ErbB2ΔIC) to the developing mouse mammary gland. Although levels of ErbB2ΔIC expression were greater than endogenous ErbB2 throughout mouse mammary gland development (data not shown), the first ErbB2ΔIC-specific phenotype, a slight defect in lobuloalveolar expansion, was observed at late gestation (19 days *post-coitus*). At parturition, however, the majority of the condensed alveoli of ErbB2ΔIC expressing mice failed to form distended lactationally active lobuloalveoli. Furthermore, there was a direct concordance between high levels of ErbB2ΔIC protein expression and the lack of lobuloalveolar secretory products.

ErbB2 is the preferred heterodimer partner of activated EGFR family members (Graus-Porta *et al.*, 1997; Tzahar *et al.*, 1996), and, when mutated, can inactivate other ErbB-family receptors (Qian *et al.*, 1994). Therefore, we cannot rule out the possibility that in addition to inhibiting ErbB2 signaling, ErbB2ΔIC inactivates signaling by other members of the EGFR-family. Nevertheless, our results suggest that proper ErbB2 signaling is required for both the terminal stages of lobuloalveolar development and lactation at parturition.

A similar MMTV-driven dominant negative strategy has been used to inactivate EGFR signaling in the developing mouse mammary gland (Xie *et al.*, 1997). Dominant negative EGFR expression inhibited mammary ductal morphogenesis in the pubescent virgin mouse. Since ErbB2 is expressed within the virgin mammary gland and is the preferred heterodimer partner of ligand activated EGFR, we expected a similar phenotype in mammary glands of pubescent mice expressing ErbB2ΔIC. One possible explanation for the lack of an ErbB2ΔIC phenotype in virgin mice is that the level of ErbB2ΔIC expression may be below the threshold level necessary to dominantly inhibit endogenous ErbB2. In addition, the mosaic transgene expression observed in our experiments resulted in high levels of ErbB2ΔIC expression in only a minority of mammary epithelial cells. Growth inhibition of a subpopulation of mammary epithelial cells expressing high levels of ErbB2ΔIC could go undetected due to immigration and overgrowth by normal ducts.

Despite high levels of transgene expression, an ErbB2ΔIC phenotype was also absent during early or mid-pregnancy. This lack of phenotype during the second and most pronounced stage of mammary epithelial proliferation (Traurig, 1967b) suggests that ErbB2 signaling is also dispensable during this period of mammary epithelial proliferation. The finding that EGF injected i.p. into pregnant mice stimulates low levels of mammary gland ErbB2 tyrosine phosphorylation, relative to EGFR, further suggests a minimal role for ErbB2 at mid-pregnancy (Schroeder and Lee, 1998).

The predominant phenotype associated with expression of ErbB2ΔIC was a failure of lobuloalveolar maturation at parturition. All four EGFR family members are highly phosphorylated at this developmental stage (Schroeder and Lee, 1998) and therefore may play a role in mammary gland development at parturition. One possibility is that ErbB2 signaling is coupled to multiple receptors at parturition, and ErbB2ΔIC abrogates signaling by all four receptors at this developmental stage. Alternatively, ErbB2 signaling may be preferentially coupled to NRG1-activated ErbB3 or ErbB4 or both at parturition. Unlike most EGFR ligands, NRG1 is expressed exclusively during pregnancy and at peak levels around parturition (Yang *et al.*, 1995). When NRG1 is injected into mice at parturition, a dramatic increase in ErbB2 and ErbB3 phosphorylation is observed (Schroeder and Lee, 1998), suggesting that these two receptors are coupled

at this developmental stage. Consistent with these results, we have previously reported that NRG1 implanted within mammary glands of virgin female mice, induced the formation of alveoli with an accumulation of the lactation product β-casein (Jones *et al.*, 1996). In addition, ectopic expression and activation of ErbB2 signaling, in the absence of activated coreceptors, induced alveolar-like structures in cultured mammary epithelial cells identical to those induced by NRG1 treatment (Niemann *et al.*, 1998). Collectively, these results suggest that NRG1 plays a role in lobuloalveolar maturation and lactation by activating ErbB2 signaling at parturition.

The phenotype at parturition observed in ErbB2ΔIC expressing mice closely resembles defects in mice with gene disruptions in Stat5a or leukocyte common-antigen-related phosphatase (LAR). Independent laboratories reported that Stat5a (Liu *et al.*, 1996; Teglund *et al.*, 1998) and LAR (Schaapveld *et al.*, 1997) are required for normal lobuloalveolar maturation and lactation at parturition. The possible coupling of ErbB2 signaling with the signaling pathways of Stat5a and LAR or both, deserves further investigation.

Substantial evidence supports the mitogenic effect of deregulated ErbB2 activity in the development and progression of human breast cancer (reviewed in: Hynes and Stern, 1994). Results presented here, however, demonstrate that normal ErbB2 signaling within the mouse mammary gland contributes to epithelial differentiation and lactation at parturition. These functionally distinct responses of mammary tissue to ErbB2 activation may be due to differential activation and coupling of ErbB2 signaling with other EGFR-family members. For example, receptors linked to mitogenic signaling pathways within mammary tissue, including amplified and overexpressed *ErbB2* alone or in combination with EGFR, would result in an aggressive mammary carcinoma associated with poor prognosis, whereas coupling of ErbB2 signaling with a receptor associated with favorable prognostic indicators, such as ErbB4 (Bacus *et al.*, 1996), may indicate a less aggressive mammary tumor. Consistent with this hypothesis, our unpublished results demonstrate that ErbB4 signaling is required for mammary epithelial differentiation, and is coupled to ErbB2 signaling at parturition. Characterization of ErbB4 signaling and other gene products associated with ErbB2 activation during mammary epithelial differentiation may lead to the identification of favorable prognostic indicators associated with ErbB2 mediated mammary carcinogenesis. Furthermore, ErbB2 signaling pathways coupled to mammary differentiation could be marshaled to antagonize the mitogenic effect of activated ErbB2 within an aggressively proliferating tumor.

Materials and methods

Plasmids

The plasmid pMMTV-ErbB2ΔIC containing the rat *ErbB2* cDNA with a carboxyl-terminal truncation at residue 694 and an HA epitope-tag (Kolodziej and Young, 1991) fused with the mouse mammary tumor virus (MMTV) LTR (Figure 1) was constructed in two steps. First, the intermediate plasmid, pN-DID-HA, was generated by trimolecular ligation of *Eco*RI digested pcDL-SRα296 (Takebe *et al.*, 1988), the 2.4 kb *Eco*RI-*Nde*I fragment from the rat *ErbB2* cDNA (Bargmann *et al.*, 1986), and the 236 bp *Nde*I–*Eco*RI digested PCR product generated from the rat *ErbB2* cDNA using a forward oligonucleotide primer upstream of the unique *Nde*I site and the reverse primer 5′ GAATGAAT TCAGGCGTAATCAGGCACATCGTATGGGTACAGCC-TACGCATCGTATAC. The modified rat *ErbB2* cDNA was then subcloned, to generate the plasmid pMMTV-ErbB2ΔIC, via a trimolecular ligation involving *Hind*III–*Eco*RI digested pMMTV-Sv40-Bssk (pMMTV-GAL4/236-SV40 minus the *GAL4*/236 gene) (Ornitz *et al.*, 1991) generously provided by Philip Leder, the 530 bp *Hind*III–*Aat*II digested PCR product generated from pN-DID-HA using the forward primer with a 5′ *Hind*III linker 5′-CTAAGCTTCAATGAT-CATCATGGAGCT-3′ and the reverse primer 5′-GGGGCA-CAAGGTGGACAGGC-3′, and the 3′ ca. 1.6 kb *Aat*II–*Eco*RI fragment from pN-DID-HA.

The plasmid pBI-ErbB2ΔIC, which served as a riboprobe template was generated as follows. The 236 bp 3′ *Nde*I (site filled with T4 DNA pol)-*Eco*RI fragment from pMMTV-ErbB2ΔIC was ligated to *Sma*I-*Eco*RI digested pBluescript I S/K (Stratagene) destroying the *Nde*I and *Sma*I sites in the process.

Immunoprecipitation and Western blot analysis

FR3T3 cell lines stably expressing ErbB2ΔIC, were produced by calcium phosphate transfection of 5 μg of pMMTV-ErbB2ΔIC together with 0.5 μg of pLXSN (Miller and Rosman, 1989), which carries a neomycin resistance gene. Transfected cells were selected in 1 mg/ml of G418 and maintained in 200 μg/ml. Stable cell clones in 60 mm dishes were metabolically labeled, in the presence of 1 μM dexamethasone, by incubating for 16 h with 100 μCi of ^{35}S-methionine/cysteine *in vitro* cell labeling mix (Amersham). Labeled cells were lysed on ice in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, with 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin) and immunoprecipitations were performed by adding 1 μg of anti-c-neu Ab-4 (Oncogene Science) and 50 μl of preswollen protein A sepharose (Pharmacia) (Stern *et al.*, 1986). Precipitated proteins were resolved by SDS–PAGE on a 12% acrylamide gel and the resolving gel was processed in Optifluor (National Diagnostics) according to the manufacturer's instructions.

For phosphotyrosine and ErbB2 Western blot analysis FR3T3 cell lines expressing ErbB2ΔIC or containing empty vector in 100 mm dishes were incubated in growth media containing 0.2% calf serum for 12 h. The cells were washed once with phosphate buffered saline (PBS; 15 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.2) and mock or EGF treated for 10 min at room temperature by adding 2.0 ml of PBS or 2.0 ml of PBS containing 250 ng of human recombinant EGF (Collaborative Biomedical), respectively. Treated cells were lysed on ice in RIPA buffer containing 1 mM pervanadate. ErbB2 was immunoprecipitated from lysate containing 300 μg of protein and analysed by SDS-PAGE as described above. Phosphotyrosine Western blot was performed using mouse monoclonal antibody 4G10 (Upstate Biotechnology) diluted to 1 μg/ml using procedures described previously (Stern *et al.*, 1986). The blot was stripped of primary antibody as described by the manufacturer (Pierce) and was reprobed for ErbB2 with Neu antibody C-18 (Santa Cruz) diluted to 0.2 μg/ml.

Generation of MMTV-ErbB2ΔIC transgenic mice

For microinjection, pMMTV-ErbB2ΔIC was digested at unique *Sal*I–*Spe*I sites and the circa 6.3 kb fragment containing the MMTV LTR, a 600 bp 5′ untranslated

region of c-Ha-*ras*, the truncated rat *ErbB2* cDNA with carboxyl-terminal HA epitope-tag, and SV40 3′ mRNA processing signals, was separated from vector sequences by agarose gel electrophoresis followed by purification with a gel extraction kit (Qiagen). The DNA fragment was microinjected into the pronuclei of B6SJL/F2 fertilized one-cell zygotes at a concentration of 12 μg/ml in 10 mM Tris, pH 7.5, 0.1 mM EDTA by Carole Pelletier, under the direction of Dr David Brownstein, at the Transgenic Mouse Shared Resource of the Yale University School of Medicine.

Identification of transgenic mice by polymerase chain reaction (PCR)

Transgenic progeny were identified by PCR analysis of DNA isolated from tail biopsies. Briefly, tail segments biopsied from 3-week-old weaned mice were incubated overnight at 55°C in 50 mM Tris, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, and 500 μg/ml proteinase K and extracted with phenol/chloroform before ethanol (EtOH) precipitation. PCR analysis using 32 cycles at 94°C for 1 min 15 s, 56°C for 2 min 15 s, and 72°C for 3 min 15 s in a PTC-100 programmable thermal cycler (M J Research, Inc.) was performed with 2 μg of tail DNA in a final volume of 100 μl containing 10 mM Tris, pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide, 0.15 μM of each primer, and 2.5 U *Taq* polymerase (Boehringer Mannheim). Primers for the amplification of a 763 bp MMTV-ErbB2ΔIC fragment were 5′ GGCCTGGGGATCCACTCGCTGGGG, corresponding to nucleotides 1364 to 1388 of the rat *ErbB2* ORF (Bargmann *et al.*, 1986) and 5′ GGCGTAATCAGGCACATCGTATGG which hybridizes to the 3′ terminal HA 12CA5 epitope of MMTV-ErbB2ΔIC. Primers for the amplification of a ca. 500 bp mouse β-casein exon 7 fragment served as a PCR internal control (Li *et al.*, 1997).

Riboprobe synthesis and purification

Where possible, buffers used for riboprobe synthesis and transcript purification were pretreated with diethyl pyrocarbonate (DEPC) using standard procedures. For RNase protection assay (RNPA) the DNA template was linearized and contaminating ribonucleases were inactivated by protease treatment at 37°C for 1 h in 10 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA, 0.6% SDS, and 150 μg/ml protease K. *In vitro* transcription and subsequent DNase treatment were performed using a MAXIscript *in vitro* transcription kit (Ambion) with 1 μg of template DNA exactly as described by the manufacturer. Full-length transcripts fractionated on a 6% polyacrylamide, 8 M urea gel, were excised, and eluted into 700 μl of 500 mM NH_4OAc, 1 mM EDTA, and 0.1% SDS at 37°C for 3 h.

RNA isolation and RNase protection assay

For isolation of mammary gland RNA, 0.2 g of tissue from the number 4 inguinal mammary gland was snap frozen in liquid N_2 and stored at −70°C until use. RNA was isolated from frozen tissue by homogenization in 4.0 ml of TRIzol (Gibco-BRL) with a glass homogenizer using a type B pestle (Janke and Kunkel KIKA-Labortechnik) using the exact procedure as described by the manufacturer. Isolated RNA was resuspended into DEPC (Sigma) treated ddH_2O and stored at −70°C until use.

For RNPA analysis, 20 μg of total RNA was precipitated with 5×10^5 c.p.m. of riboprobe. The same amount of t-RNA (Gibco-BRL) precipitated with riboprobe served as a negative control. Riboprobe hybridization, RNA digestion, and isolation of RNA/probe hybrids, were performed using the RPA II ribonuclease protection assay kit (Ambion) exactly as described by the manufacturer. Probe protected RNA fragments were separated on a 6% polyacrylamide 8 M urea gel and, without additional processing, the gel was exposed to XAR-5 (Kodak) x-ray film at −70°C for 24–48 h.

Whole-mount staining of mouse mammary glands

The entire number 4 inguinal mammary gland was spread onto a glass microscope slide, fixed in acidic ethanol and stained in carmine solution exactly as described previously (Jones *et al.*, 1996).

Tissue preparation for histological analysis

For hematoxylin/eosin staining and immunohistochemistry (IHC) a portion of the number 4 inguinal mammary gland was spread onto a glass microscope slide and fixed in freshly prepared 4% paraformaldehyde in PBS (15 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.2) overnight at 4°C. The fixed tissue was rinsed in ddH_2O and partially dehydrated through an ascending EtOH series to 70%. The samples were embedded in paraffin and 6 μm sections were dried onto gelatin-coated slides using standard procedures.

Immunohistochemistry

For immunohistochemical detection of HA-tagged dominant negative ErbB2, paraffin embedded mammary glands were dewaxed in xylene, hydrated through a descending EtOH series, and washed in PBS. Endogenous peroxidase activity was inactivated by incubating the sections in 0.5% H_2O_2 in PBS for 15 min at room temperature followed by two washes in PBS for 5 min per wash. Non-specific binding sites were blocked for 30 min at room temperature with 10% rabbit serum in PBS. Sections were sequentially incubated first with 50 μl of 10% rabbit serum in PBS containing 1 μg of anti-HA high affinity rat monoclonal antibody (Boehringer Mannheim) overnight at 4°C, then 50 μl of 10% rabbit serum in PBS containing 1 μg of biotinylated goat anti-rabbit IgG (Vector) for 1 h. at room temperature, and finally 50 μl biotin-avidin-peroxidase complex generated in PBS using a Vectastain Elite ABC kit (Vector) for 30 min at room temperature. Between each reagent, the sections were washed three times in PBS for 15 min per wash. Peroxidase activity was detected by incubating the sections for 7 min with 200 μl of DAB substrate (Vector). Negative controls included similarly processed mammary gland paraffin sections from a non-transgenic sibling at 1 day *post-partum* and sections from ErbB2ΔIC-expressing mammary glands processed without anti-HA primary antibody. Sections were lightly counterstained in hematoxylin (Polysciences), dehydrated through an ascending EtOH series, cleared in xylene, and coverslipped with permount (Fisher).

Acknowledgments

We thank Catrina Dompé and Christine Burke for construction of pN-DID-HA and Brian Jones for transgene purification. We thank Elizabeth Kuklinska of the Yale University Critical Technologies Program for preparation of paraffin tissue sections and hematoxylin/eosin staining. We thank JoAnn Falato for expert administrative assistance. We thank Jonathan McMenamin-Balano and Marc Schwartz for critical reading of this manuscript. We thank Debbie Colditz and Rajani Ramabhadran for excellent technical support and other members of the Stern lab for advice and critical insights.

This work was supported by United States Public Health Service grant RO1CA45708 from the National Cancer Institute, and F Jones was supported by US Army Medical Research and Material Command grant DAMD17-96-1-6158.

References

Bacus SS, Huberman E, Chin D, Kiguchi K, Simpson S, Lippman M and Lupu R. (1992). *Cell Growth Differ.*, **3**, 401–411.

Bacus SS, Chin D, Yarden Y, Zelnick CR and Stern DF. (1996). *Am. J. Pathol.*, **148**, 549–558.

Bargmann CI, Hung M-C and Weinberg RA. (1986). *Nature*, **310**, 226–230.

Chen X, Levkowitz G, Tzahar E, Karunagaran D, Lavi S, Ben-Baruch N, Leitner O, Ratzkin BJ, Bacus SS and Yarden Y. (1996). *J. Biol. Chem.*, **271**, 7620–7629.

Coleman S, Silberstein GB and Daniel CW. (1988). *Dev. Biol.*, **127**, 304–315.

Coussens L, Yang-Feng TL, Liao Y-C, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, Levinson A and Ullrich A. (1985). *Science*, **230**, 1132–1139.

DiAugustine RP, Richards RG and Sebastian J. (1997). *J. Mam. Gland Biol. Neoplasia*, **2**, 109–117.

Dickson RB and Lippman ME. (1995). *Endocrine Rev.*, **16**, 559–589.

DiFiore PP, Segatto O, Taylor WG, Aaronson SA and Pierce JH. (1990). *Science*, **248**, 79–83.

Ederly M, Pang K, Larson L, Colosi T and Nandi S. (1985). *Endocrinology*, **117**, 405–411.

Graus-Porta D, Beerli RR, Daly JM and Hynes NE. (1997). *EMBO J.*, **16**, 1647–1655.

Hynes NE and Stern DF. (1994). *Biochim. Biophys. Acta.*, **1198**, 165–184.

Jones FE, Jerry DJ, Guarino BC, Andrews GC and Stern DF. (1996). *Cell Growth Differ.*, **7**, 1031–1038.

Karunagaran D, Tzahar E, Beerli RR, Chen X, Graus-Porta D, Ratzkin BJ, Seger R, Hynes NE and Yarden Y. (1996). *EMBO J.*, **15**, 254–264.

Kenney NJ, Huang R-P, Johnson GR, Wu J-X, Okamura D, Matheny W, Kordon E, Gullick WJ, Plowman G, Smith GH, Salomon DS and Adamson ED. (1995). *Mol. Repro. Devel.*, **41**, 277–286.

Kenney NJ, Smith GH, Rosenberg K, Cutler ML and Dickson RB. (1996). *Cell Growth Differ.*, **7**, 1769–1781.

Kokai Y, Meyers JN, Wada T, Brown VI, LeVea CM, Davis JG, Dobashi K and Greene MI. (1989). *Cell*, **58**, 287–292.

Kolodziej PA and Young RA. (1991). *Methods Enzymol.*, **194**, 508–519.

Kraus MH, Issing W, Miki T, Popescu NC and Aaronson SA. (1989). *Proc. Natl. Acad. Sci. USA*, **86**, 9193–9197.

Li B, Rosen JM, McMenamin-Balano J, Muller WJ and Perkins AS. (1997). *Mol. Cell. Biol.*, **17**, 3155–3163.

Liu X, Robinson GW, Wagner K-U, Garrett L, Wynshaw-Boris A and Hennighausen L. (1996). *Genes Dev.*, **11**, 179–186.

Miller AD and Rosman GJ. (1989). *Biotechniques*, **7**, 980–990.

Nass SJ, Hahm HA and Davidson NE. (1998). *Nature Med.*, **4**, 761–762.

Niemann C, Brinkman V, Spitzer E, Hartmann G, Sachs M, Naundorf H and Birchmeier W. (1998). *J. Cell Biol.*, **143**, 533–545.

Ornitz DM, Moreadith RW and Leder P. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 698–702.

Parker SL, Tong T, Bolden S and Wingo PA. (1996). *CA-Cancer J. Clin.*, **65**, 5–27.

Peles E, Bacus SS, Koski RA, Lu HS, Wen D, Ogden SG, Ben Levy R and Yarden Y. (1992). *Cell*, **69**, 205–216.

Plowman GD, Culouscou J-M, Whitney GS, Green JM, Carlton GW, Foy L, Neubauer MG and Shoyab M. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 1746–1750.

Qian X, Dougall WC, Hellman ME and Greene MI. (1994). *Oncogene*, **9**, 1507–1514.

Qian X, O'Rourke DM, Zhao H and Greene MI. (1996). *Oncogene*, **13**, 2149–2157.

Revillion F, Bonneterre J and Peyrat JP. (1998). *Eur. J. Cancer*, **34**, 791–808.

Riese II DJ, Komurasaki T, Plowman GD and Stern DF. (1998). *J. Biol. Chem.*, **273**, 11288–11294.

Riese II DJ and Stern DF. (1998). *BioEssays*, **20**, 41–48.

Salomon DS, Brandt R, Ciardiello F and Normanno N. (1995). *Crit. Rev. Oncol.*, **19**, 183–232.

Schaapveld RQJ, Schepens JTG, Robinson GW, Attema J, Oerlemans FTJJ, Fransen JAM, Streuli M, Wieringa B, Hennighausen L and Hendricks WJAJ. (1997). *Dev. Biol.*, **188**, 134–146.

Schroeder JA and Lee DC. (1998). *Cell Growth Differ.*, **9**, 451–464.

Sebastian J, Richards RG, Walker MP, Wiesen JF, Werb Z, Derynck R, Hom YK, Cunha GR and DiAugustine RP. (1998). *Cell Growth Differ.*, **9**, 777–785.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL. (1987). *Science*, **235**, 177–182.

Snedeker SM, Brown CF and DiAugustine RP. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 276–280.

Spivak-Kroizman T, Rotin D, Pinchasi D, Ullrich A, Schlessinger J and Lax I. (1992). *J. Biol. Chem.*, **12**, 8056–8063.

Stern DF, Heffernan PA and Weinberg RA. (1986). *Mol. Cell. Biol.*, **6**, 1729–1740.

Stern DF and Kamps MP. (1988). *EMBO Journal*, **7**, 995–1001.

Takebe Y, Seiki M, Fujisawa J-I, Hoy P, Yokota K, Arai K-I, Yoshida M and Arai N. (1988). *Mol. Cell. Biol.*, **8**, 466–472.

Teglund S, McKay C, Schuetz E, vanDeursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G and Ihle JN. (1998). *Cell*, **93**, 841–850.

Topper YJ and Freeman CS. (1980). *Physiological Rev.*, **60**, 1049–1106.

Traurig HH. (1967a). *Anat. Rec.*, **157**, 489–504.

Traurig HH. (1967b). *Anat. Rec.*, **159**, 239–248.

Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, Ratzkin BJ and Yarden Y. (1996). *Mol. Cell. Biol.*, **16**, 5276–5287.

Vonderhaar BK. (1984). *Control of cell growth and proliferation*. Veneziale, CM (ed). van Nostrand-Reinhold: Princeton, NJ, pp. 11–33.

Wada T, Qian X and Greene MI. (1990). *Cell*, **61**, 1339–1347.

Xie W, Paterson AJ, Chin E, Nabell LM and Kudlow JE. (1997). *Mol. Endocrinol.*, **11**, 1766–1781.

Yang Y, Spitzer E, Meyer D, Sachs M, Niemann C, Hartmann G, Weidner KM, Birchmeier C and Birchmeier W. (1995). *J. Cell Biol.*, **131**, 215–226.

Yoo J-Y and Hamburger AW. (1998). *Mol. Cell. Endocrinol.*, **138**, 163–171.

Heregulin Induces *in Vivo* Proliferation and Differentiation of Mammary Epithelium into Secretory Lobuloalveoli[1]

Frank E. Jones, D. Joseph Jerry, Brad C. Guarino, Glenn C. Andrews, and David F. Stern[2]

Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520-8023 [F. E. J., D. F. S.]; Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts 01003-6410 [D. J. J.]; and Pfizer Central Research, Groton, Connecticut 06340 [B. C. G., G. C. A.]

Abstract

Mammary gland development and differentiation is mediated through the combined activities of systemic hormones and locally synthesized growth factors. To determine the *in vivo* response of mammary epithelium to heregulin (HRG), we implanted Elvax pellets containing HRGα or HRGβ within the mammary glands of prepubescent female mice in the presence or absence of exogenous estradiol and progesterone (E/P). Mice treated in the same way with transforming growth factor α (TGF-α) were included as a positive control. Each growth factor treatment induced epithelial ductal branching in the presence or absence of E/P. In the absence of E/P, HRGβ did not affect terminal end bud formation, mammary epithelium branching, or ductal migration. In contrast, TGF-α and HRGα induced ductal branching and HRGα induced ductal migration in the absence of E/P. The overall mammary response to growth factors was potentiated by the concomitant presence of E/P. In every case, the *in vivo* mammary epithelial responses to HRGα were more robust than TGF-α. Limited lobuloalveolar development was also observed in growth factor-treated mammary glands when E/P was present. Histological examination of growth factor-induced lobuloalveoli revealed secretory products within the lumen of HRGα and HRGβ lobuloalveoli. TGF-α-induced lobuloalveoli lacked similar secretory products.

Introduction

Mammary gland development is unusual in that the vast majority of growth and differentiation occurs postnatally. In the prepubescent mouse, mammary ductal structures emanating from the nipple terminate in large bulbous structures referred to as TEBs.[3] With the onset of puberty, steroid hormones function as potent mitogens of TEB. This rapidly dividing cell population is responsible for ductal growth and branching during expansion of the mammary gland. During pregnancy, an additional pronounced growth cycle results in increased ductal branching and lobuloalveolar development. The lobuloalveoli terminally differentiate into milk-producing structures, and the extensive lobuloalveoli completely fill the interductal spaces during lactation (1, 2). These developmental processes are regulated through a complex series of events requiring the activities of both intraglandular and systemic hormones/growth factors (3–5). The steroid hormones estrogen and progesterone are major players in these developmental processes. However, the exact mechanisms underlying steroid hormone growth effects are not known and may involve a combination of direct effects and/or stimulation of growth factors which in turn mediate mammary gland development in a juxtacrine or autocrine fashion. Indeed, estradiol stimulates mammary epithelial expression and/or secretion of several EGF family members (6–10), and these growth factors have several important functional roles during mammary gland development (4, 5, 11).

Normal breast tissue expresses several EGF family members including EGF (12), TGF-α (12–16), amphiregulin (16–18), cripto-1 (16–18), and HRG (19). In addition, mammary gland expression of all four EGFR family members identified to date (*e.g.*, EGFR, erbB-2/HER-2/neu, erbB-3, and erbB-4) has been reported (19–22). A substantial body of evidence suggests that the EGF family of growth factors and their cellular receptors play an important role in both normal and malignant mammary gland development (4, 5, 11, 23–27). Most recently, the function of HRG in mammary gland development has been investigated. In mammary tumor cells, HRGs appear to have a mitogenic effect (28–31) or induce differentiation of mammary epithelium with the synthesis of milk proteins (30, 32–34). Yang *et al.* (19) examined the affects of HRG on mammary gland morphogenesis. In whole-organ culture, HRG stimulates lobuloalveolar development and the production of milk proteins. A putative role for HRG in lobuloalveolar development and milk production is further supported by the following observations; HRGα is expressed within the mammary mesenchyme adjacent to lobuloalveolar structures, and HRGα expression is regulated during mammary gland development and is only expressed during pregnancy (19). Therefore, HRG appears to be a po-

Received 3/25/96; accepted 6/6/96.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by USPHS Grant CA-45708 from the National Cancer Institute and by Grant DAMD-17-94-J-4476 from the U. S. Army Research and Material Command (to D. F. S.). Partial support was supplied by Grant SCDPH3408508C013 from the Massachusetts Department of Public Health (to D. J. J.). F. E. J. is supported by USPHS Grant T32DK07556.
[2] To whom requests for reprints should be addressed. Phone: (203) 785-4832; Fax: (203) 785-7467; E-mail: stern@biomed.med.yale.edu.

[3] The abbreviations used are: TEB, terminal end bud; EGF, epidermal growth factor; EGFR, EGF receptor; HRG, heregulin; TGF-α, transforming growth factor α; E/P, estradiol and progesterone; RP-HPLC, reverse phase high pressure liquid chromatography.

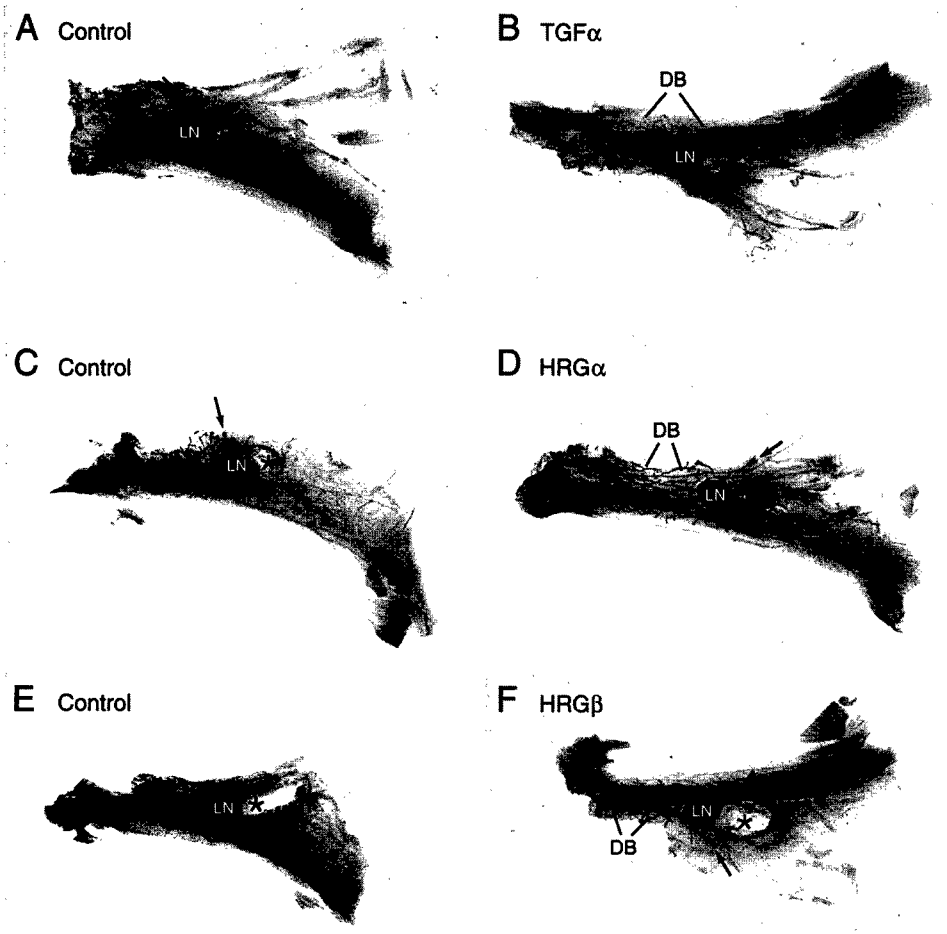


Fig. 1. Effect of growth factor treatment on mammary gland morphology in the absence of estradiol and progesterone. Control Elvax pellets and pellets containing growth factor were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Implants were positioned (*) immediately anterior to the central lymph node (*LN*). Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). Terminal end buds are indicated by *arrows*, and regions of growth factor-induced ductal branching (*DB*) are indicated. Implants contained 10 μg of TGF-α (*B*), 5 μg of HRGα (*D*), and 10 μg of HRGβ (*F*). Contralateral control for each sample is represented (*A*, *C*, and *E*).

tent and developmentally important mammary epithelial growth factor.

Despite these recent observations, the role of HRG in mammary gland morphogenesis *in vivo* has not been investigated. To address this issue, we have used a mouse model system to examine the direct effects of HRG on mammary epithelium under conditions similar to those where this growth factor normally functions. The mammary glands of prepubescent female mice were surgically implanted with Elvax pellets containing recombinant HRGα, HRGβ, or TGF-α in the presence or absence of steroid hormones. All three growth factors possessed differing levels of epithelial mitogenic activity *in vivo*. In addition, in the presence of steroid hormones, each growth factor induced epithelial differentiation into lobuloalveolar structures. However, only the HRG-treated lobuloalveoli underwent terminal differentiation, resulting in the lumenal accumulation of secretory products. Taken together, these experiments offer the first *in vivo* evidence for a role of HRG in mammary epithelial development and terminal differentiation into milk protein-secreting lobuloalveolar structures.

Results

HRG Induces Ductal Branching *in Situ*. HRG induces pleiotropic responses in cultured mammary epithelial cells (19, 28, 29, 31, 33–35); however, the *in vivo* response of mammary epithelium to this family of growth factors has not been investigated. As a first step toward identifying a biological role for HRG in mammary ductal morphogenesis, we surgically implanted slow-release pellets containing varying amounts of HRGα or HRGβ within the developing mammary fat pad of virgin female mice. HRGα and HRGβ are splice variants that possess differing EGF domains (36). Pellets lacking growth factor were inserted into the contralateral fat pad as a negative control. Previously, TGF-α has been shown to induce ductal branching and lobuloalveolar development in a similar experimental system (37) and was, therefore, included as a positive control in our experiments. The mice were sacrificed 3 days after implant insertion, and whole mounts of the mammary glands were examined for ductal morphogenesis and lobuloalveolar development.

When compared to contralateral controls, each growth factor induced ductal branching within the treated mammary gland (Figs. 1 and 2). Responses to growth factors in the

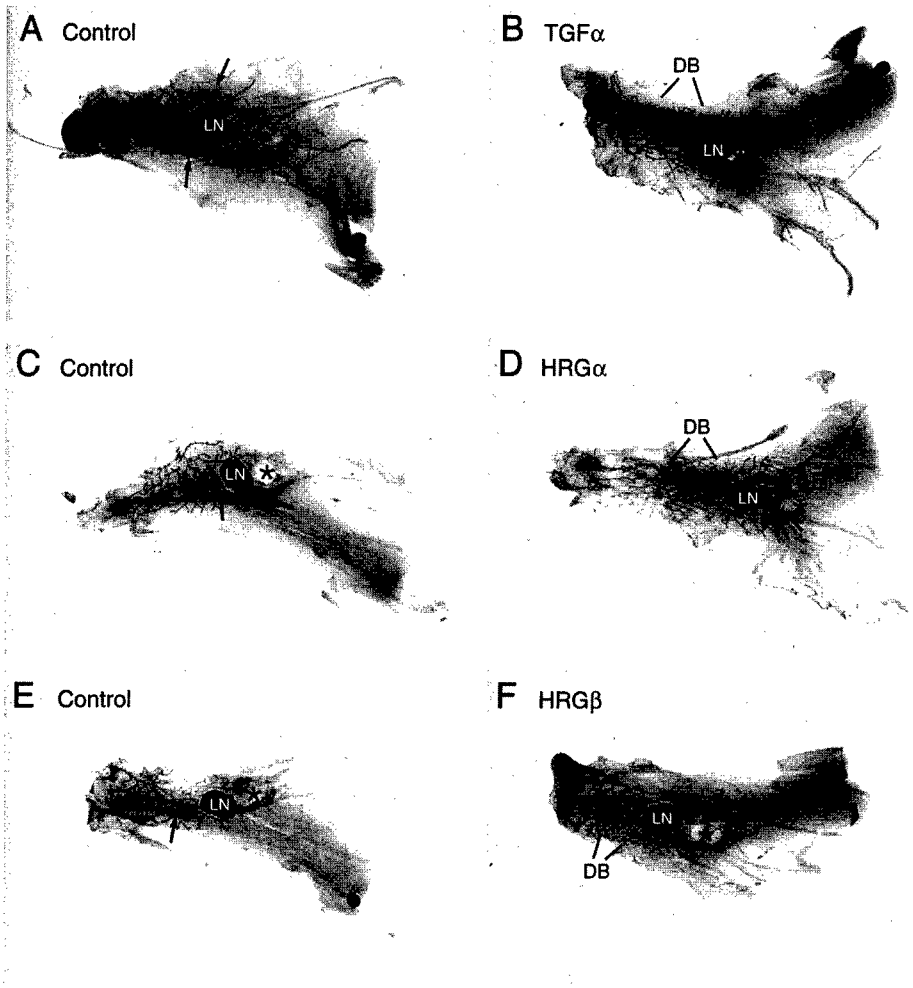


Fig. 2. Effect of growth factor treatment on mammary gland morphology in the presence of estradiol and progesterone. Control Elvax pellets containing 10 μg of 17 β-estradiol and 1 mg of progesterone (E/P) and pellets containing growth factor with E/P were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Implants were positioned (*) immediately anterior to the central lymph node (*LN*). Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). Terminal end buds are indicated by *arrows*, and regions of growth factor-induced ductal branching (*DB*) are indicated. Implants contained 10 μg of TGF-α (*B*), 5 μg of HRGα (*D*), and 10 μg of HRGβ (*F*). Paired contralateral control for each sample is represented (*A, C,* and *E*).

presence or absence of E/P were maximal with pellets containing 10 μg of TGF-α or HRGβ or 5 μg of HRGα (data not shown). These growth factor concentrations were used in all subsequent experiments. In the absence of E/P, each growth factor induced ductal branching in the region posterior to the central lymph node (Fig. 1, compare *panels A* to *B*, *C* to *D*, and *E* to *F*). However, differences between each growth factor could be identified. For example, the ductal branching observed in HRGβ-treated glands (Fig. 1*F*) was less extensive than glands treated with either TGF-α (Fig. 1*B*) or HRGα (Fig. 1*D*). In addition, where TGF-α and HRGβ treatment appeared to inhibit TEB formation (Fig. 1, *B* and *F*, respectively), HRGα not only induced TEB proliferation but also increased ductal migration anterior to the central lymph node (Fig. 1*D*).

Treatment of control mammary glands with E/P alone resulted in a slight increase in ductal diameter (compare Fig. 1*A* to Fig. 2*A*). Moreover, the mammary response to growth factors was potentiated by the presence of E/P because ductal branching induced by each growth factor was more pronounced in the presence of E/P (compare Fig. 1, *B, D,* and *F,* to Fig. 2, *B, D,* and *F,* respectively). The mammary epithelial responses to implants containing TGF-α (Fig. 2*B*) and HRGβ (Fig. 2*F*) were similar because both growth factors inhibited TEB formation. In contrast, HRGα induced TEB formation, and the overall epithelial response to HRGα was more robust (Fig. 2*D*) than either TGF-α (Fig. 2*B*) or HRGβ (Fig. 2*F*).

The extent of ductal branching, ductal growth, and TEB formation induced by each growth factor in the presence and absence of E/P was quantitated. Data from 10 mice, for each experimental condition, was subjected to statistical analysis. Due to high variability among mice, each quantitated parameter was normalized to the contralateral control within an individual animal. Although each growth factor induced ductal branching (Figs. 1 and 2), branching induced by HRGβ was statistically significant only in the presence of E/P (Fig. 3). In general, HRGα appeared to induce a more robust and pleiotropic response within treated mammary glands than either TGF-α or HRGβ. Indeed, ductal branching was more extensive in HRGα-treated glands whether in the presence or absence of E/P (Fig. 3). Moreover, whereas TGF-α and HRGβ appeared to slightly inhibit or had no affect on TEB formation, HRGα induced TEB proliferation in the presence of E/P (Fig. 3). Moreover, HRGα was the only growth factor to significantly increase ductal length within treated mammary glands

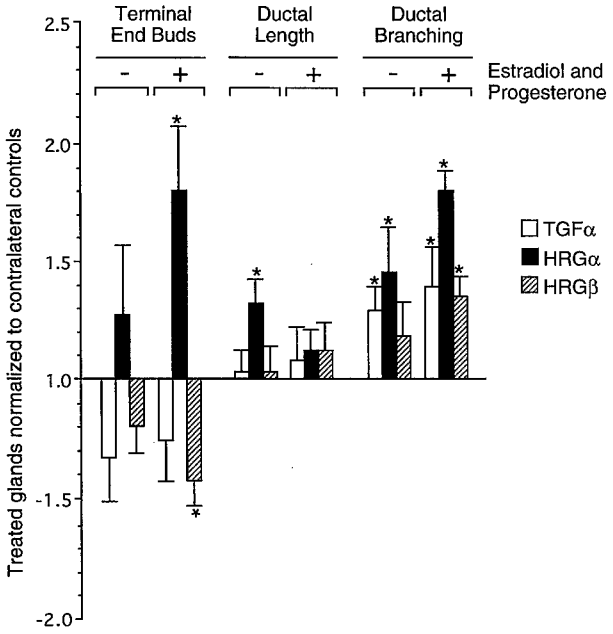


Fig. 3. Effects of growth factors on mammary epithelium. Ten 30-day-old female BALB/c mice were implanted with growth factor pellets at the growth factor's concentration of maximal response in the absence (−) or presence (+) of 10 μg of estradiol and 1 mg of progesterone. Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with carmine (see "Materials and Methods"). The number of TEBs, the ductal length of the longest duct per fat pad, and the number of ductal branches was determined for each treated and contralateral control gland. Treated glands were normalized to their contralateral controls, and the means plus 1 SD (*bars*) are represented above. Growth factor-induced phenotypes significantly different from contralateral controls (paired student *t* test; $P < 0.01$) are indicated (*). Implants contained 10 μg of TGF-α, 5 μg of HRGα, and 10 μg of HRGβ.

(Fig. 3). With the exception of increased ductal length induced by HRGα in the absence of E/P, the concomitant presence of E/P appeared to potentiate the mammary epithelial response to each growth factor tested (Fig. 3).

HRG Induces Mammary Lobuloalveolar Development and the Accumulation of Lumenal Secretory Products *in Vivo*. Whole mounts prepared from growth factor-treated mammary glands revealed extensive epithelial ductal branching. We next examined the terminal ducts within treated and control mammary glands for evidence of lobuloalveolar structures. As expected, untreated glands or glands treated with E/P alone did not develop lobuloalveolar structures. In contrast, a limited extent of lobuloalveolar development was observed in a majority of mammary glands treated with growth factor (Fig. 4, *A-C*). Growth factor-induced lobuloalveoli required the concomitant presence of E/P because similar structures were not observed in glands treated with growth factors alone. Consistent with previous results, the effect of HRGα was more robust than either TGF-α or HRGβ. HRGα induced lobuloalveolar development in 77% of treated mammary glands, whereas TGF-α and HRGβ induced lobuloalveoli in 39 and 46% of treated glands, respectively (13 glands were examined for each treatment). Histological examination of growth factor-induced lobuloalveoli revealed numerous epithelial buds typical of these structures (Fig. 4, *D-F, arrows*). In addition, HRGα- and HRGβ-induced lobuloalveoli exhibited accumulation of lumenal secretory products (Fig. 4, *E* and *F*), which stained positive for β-casein by immunohistochemistry (data not shown). Similar accumulations were not observed in TGF-α-induced lobuloalveoli (Fig. 4*D*).

Discussion

Mammary gland development involves a complex and highly regulated sequence of postnatal events. Recently, expression of an EGF-related subfamily of growth factors termed the neu differentiation factors or HRGs was detected *in vivo* within connective tissue juxtaposed to fully differentiated, milk-secreting lobuloalveoli (19). To determine if HRG plays a role in mammary epithelial growth and/or differentiation *in vivo*, we inserted slow-release pellets containing HRG within mammary glands of prepubescent mice and analyzed the *in vivo* response of mammary epithelium to these growth factors. We found that HRGα and HRGβ induced epithelial branching and differentiation into lobuloalveolar structures, as does a related growth factor, TGF-α. However, histological examination of TGF-α- and HRG-induced lobuloalveoli revealed a striking difference; HRGα and HRGβ stimulated the accumulation of lumenal secretory products, including the milk protein β-casein, within treated lobuloalveoli. TGF-α-induced lobuloalveoli lacked similar lumenal accumulations. These results suggest that HRG can induce terminal differentiation of mammary epithelium *in vivo* into milk protein-secreting lobuloalveolar structures.

The epithelial response to growth factor implants was potentiated by the concomitant presence of estradiol and progesterone. Indeed, lobuloalveoli were only observed in the presence of these steroid hormones. A similar requirement of estradiol and progesterone for EGF- and HRG-induced lobuloalveoli in mammary organ culture has been reported (4, 19, 38). Some evidence suggests that the requirement of exogenous steroid hormones may also reflect strain differences. For example, TGF-α implants induce lobuloalveoli development in CH3/HeN mice in the absence of exogenous estradiol and progesterone (37). In contrast, our results indicate that induction of lobuloalveoli by TGF-α or HRG in BALB/c mice requires the concomitant presence of exogenous estradiol and progesterone. Although we did not perform experiments to determine if estradiol or progesterone alone could augment mammary responses to growth factors, substantial evidence indicates that both estradiol and progesterone have independent proliferative effects on mammary epithelium. Furthermore, co-administration of these hormones enhances independent proliferative effects (3). Using mice carrying a null mutation in the progesterone receptor, Lydon *et al.* (39) demonstrated the *in vivo* requirement of progesterone in ductal epithelium proliferation and lobuloalveoli differentiation. Thus, it seems probable that progesterone contributes to the growth factor-induced lobuloalveolar development observed in our experiments. Although estradiol is considered to be the steroid hormone most directly involved in mammary epithelial proliferation (3), the exact role of estradiol in mammary development is poorly defined. The

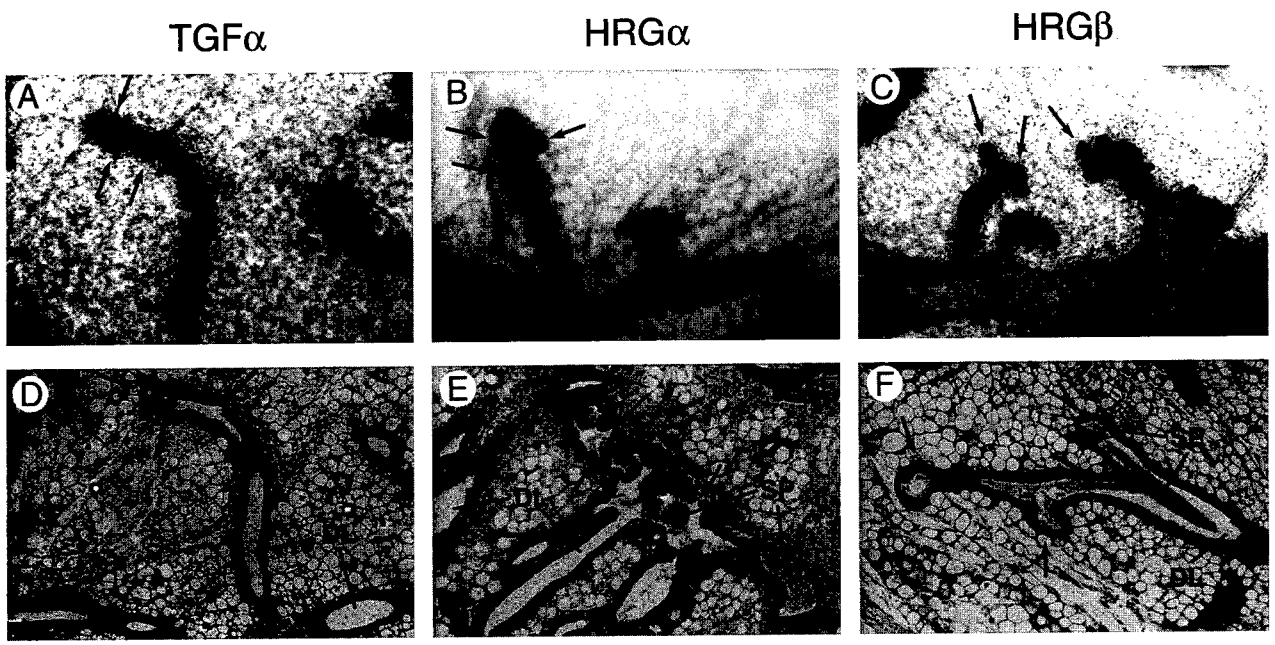


Fig. 4. Growth factor-induced lobuloalveolar development in prepubescent female mice. Elvax pellets containing growth factor with 10 μg of 17 β-estradiol and 1 mg of progesterone were implanted within the surgically exposed number 4 inguinal mammary fat pad of 30-day-old female BALB/c mice. Mice were sacrificed 3 days following implant insertion, and excised mammary glands were processed using the whole-mount procedure and stained with hematoxylin (see "Materials and Methods"). Photomicrographs of paraffin sections revealed lobuloalveolar structures in growth factor-treated glands (*A-C*). *Arrows* indicate epithelial buds typical of these structures. Histological examination of paraffin-embedded lobuloalveoli stained with H&E (*D* and *E*) revealed secretory products (*SP*) within the ductal lumens (*DL*) of HRGα- and HRGβ-treated mammary glands (*E* and *F*, respectively). Implants contained 10 μg of TGF-α (*A* and *D*), 5 μg of HRGα (*B* and *E*), and 10 μg of HRGβ (*C* and *F*).

reason for this lack of clarity lies in the fact that in addition to independent proliferative effects, estradiol also induces mammary expression of several growth factors, including the EGF family members EGF (7), TGF-α (6, 7, 40), and amphiregulin (9, 41). Mammary gland expression of these EGF family members may directly affect mammary development and thereby augment the epithelial response to growth factor implants observed in our experiments.

The qualitative and quantitative differences in mammary epithelial responses to TGF-α and HRG can be explained through functional differences of the two growth factors. Histochemical analysis of mouse mammary glands reported elsewhere revealed dramatic differences in the cellular localization and expression of these growth factors. Mammary gland expression of TGF-α was detected during each epithelial developmental stage with the exception of lactation, and immunostaining within the cap cell layer of the TEB and epithelial cells of subtending ducts was observed (12). In contrast, expression of HRG is induced during pregnancy within the connective tissue adjacent to ductal and lobuloalveolar structures (19). Differing mammary epithelial responses to TGF-α and HRG may also reflect the activation of different signaling tyrosine receptor kinases within these cellular populations. TGF-α binds directly to the EGFR (26) and can activate erbB-2 (42), erbB-3, and erbB-4,[4] presumably through a ligand-driven receptor cross-phosphorylation mechanism (43, 44) also referred to as "transmodulation" (24, 45). Similarly, HRG binds directly to erbB-3 (46, 47) and erbB-4 (47) and can drive the activation of EGFR and erbB-2 (46–48). Evidence from *in vitro* experiments indicates that cellular responses to signaling by this family of receptors can be radically different, depending upon both the transmodulation partner and the activating growth factor (48–51). Therefore, one prediction follows that signaling by EGFR family members *in vivo* would also induce a diversity of cellular responses that are dependent upon the activating growth factor. Cellular responses to HRG *in vivo* appear to be regulated primarily but not exclusively through erbB-2 signaling. Disruption of HRG or erbB-2 in transgenic mice results in a similar embryonic lethal phenotype characterized by nearly identical heart malformations and neural crest development defects (52, 53). Moreover, expression patterns within the developing rhombencephalon suggest that a HRG: erbB-2 autocrine or paracrine signaling relationship has been disrupted in these mice (52, 53). These observations further support a direct relationship between HRG and erbB-2 signaling. A similar relationship may mediate HRG activity in mammary epithelium, and we are presently designing experiments to examine this possibility.

In our experiments, the *in vivo* response of mammary epithelium to HRGα was more robust than HRGβ. This result was surprising because *in vitro* experiments consistently identify HRGβ as the more potent growth factor (28, 30, 31, 33). However, we used chemically synthesized and bacterial recombinant peptides in our experiments, which may not

[4] D. J. Riese II, E. Kim, G. Allison, S. Buckley, M. Klagsbrun, G. D. Plowman, and D. F. Stern. J. Biol. Chem., in press.

represent the complete activities of full-length HRG protein. Alternatively, the enhanced mammary response to HRGα may reflect a physiological role for HRGα and not HRGβ in mammary gland development. Indeed, only HRGα isoforms are expressed in the mammary gland, and this expression is induced during pregnancy (19). Thus, the HRGα expression pattern strongly correlates with the *in vivo* function identified in this communication. Our experiments provide the first demonstration of an important *in vivo* role for HRG in mammary epithelium proliferation and differentiation into secretory lobuloalveoli. In conclusion, we propose that HRGα is the physiologically relevant HRG isoform expressed within the developing mammary gland, and HRGα plays an important role in the differentiation of mammary epithelium into milk-secreting lobuloalveoli.

Materials and Methods

Plasmid Construction. The human HRG β1 cDNA fragment corresponding to residues 177–244 (54) was subcloned into the pNB261 bacterial expression vector as follows. Poly(A) mRNA was isolated from cultured human MDAMB231 cells (American Type Culture Collection) by use of the Fast Track mRNA isolation kit (InVitrogen), according to the manufacturer's instructions. The HRG β1 cDNA fragment corresponding to residues 177–244 was amplified by a 30-cycle reverse transcription-PCR procedure using the RNA Gene Amp kit (Perkin-Elmer Corp.) and the primers incorporating 5′ *Eco*RI (sense 5′-CGCGAATTCTATGAGCCATCT-TGTAAAATGTGC) and *Hin*dIII (anti-sense 5′-CGCGAAGCTTAGTA-CAGCTCCTCCGCCTCCAT) linkers. The 204-bp amplified fragment was digested with *Eco*RI/*Hin*dIII and inserted into the same sites of the Bluescript vector pCRII (Stratagene). The nucleotide sequence of the 204-bp insert was confirmed by use of an Applied Biosystems Automated Sequencer using standard methods. The sequenced 204-bp insert was excised from pCRII by digestion with *Eco*RI/*Hin*dIII and subcloned downstream of the trp-inducible promoter using the same restriction sites of the pNB261 expression vector (construct pHerβ1ST). The sequence of the 204-bp human HRG β1 insert was confirmed as described above.

Expression and Purification of Human Recombinant HRG β1 (177–244). For large scale fermentation and expression of HRG β1 (177–244), pHERβ1ST was transformed into the *Escherichia coli* strain GE81. Bacterial cells from a 10-liter fermentation in modified M9 medium (55) were harvested by centrifugation, and expression was induced by resuspending the bacteria pellet into fresh modified M9 medium lacking tryptophan. After an induction period of 4 h, a total of 69 g of cell paste was recovered by centrifugation. Expression of the predicted 7000-Da product peaked at 3 h postinduction. A 25-g bacterial pellet was resuspended into 50 ml of lysis buffer [20 mM Tris (pH 8.0), 40 mM NaCl, 0.25 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] and passed through a French press twice. The lysate was centrifuged for 45 min at 12,000 rpm, and the pellet was resuspended into 20 ml of lysis buffer. Fifteen mg of hen egg white lysozyme (Sigma Chemical Co.) was added, and the mixture was incubated at room temperature for 20 min. Three hundred μl of 1 mg/ml DNase and 800 μl of 100 mM $MgCl_2$ were added, and the mixture was incubated at room temperature for an additional 15 min. The suspension was centrifuged for 15 min at 12,000 rpm, and the final inclusion body pellet was washed twice with 1.0% NP40 and once with ddH_2O and lyophilized to dryness to yield 500 mg of dried inclusion bodies. A suspension of 10 mg of inclusion bodies in 2.0 ml of 50 mM Tris (pH 6.0), 6 M guanidinium hydrochloride, and 200 mM DTT was heated at 37°C for 2 h and filtered through a Costar 3.5-μm spin-filter; then the filtrate was diluted into 100 ml of folding buffer [50 mM Tris (pH 8.6), 1 M urea, 1.5 mM glutathione, 0.75 mM glutathiol, and 10 mM methionine] and stirred for 5 days at 4°C. The folded, oxidized protein was isolated by RP-HPLC on a VYDAC C-4 reverse phase column using an acetonitrile/ddH_2O/0.1% trifluoroacetic acid gradient. The isolated protein was homogeneous by RP-HPLC and capillary electrophoresis, and was composed of 206 μg by amino acid analysis. The protein exhibited a mass of 7877.8 Da by electrospray mass spectrometry [theoretical mass for oxidized HRG β1 (177–244) is 7878.1 Da].

Growth Factors. HRGα 177–228 (HRGα 52) was synthesized on an Applied Biosystems 430A peptide synthesizer using standard *tert*-butyloxycarbonyl chemistry protocols provided by the manufacturer (version 1.40; *N*-methylpyrolidone/hydroxybenzotriazole). Peptide was purified by RP-HPLC, characterized by electrospray mass spectroscopy, and analyzed for disulfide bonding as described previously (56). Peptide quantities were determined by amino acid analysis. Human recombinant TGF-α was purchased from Collaborative Biomedical Products.

Implant Preparation. Growth factor peptides and steroid hormones were encapsulated within Elvax pellets essentially as described elsewhere (57). Briefly, a lyophilized mixture containing growth factor peptide and, where indicated, the steroid hormones 17-β-estradiol (10 μg; Sigma) and progesterone (1 mg; Sigma) was suspended in 25 μl of Elvax (generously donated by Elf Atochem, Philadelphia, PA) dissolved previously in dichloromethane (15% w/v). The entire suspension was transferred to an Eppendorf tube, snap-frozen in liquid nitrogen, and dried under vacuum. The dried Elvax pellet was compressed between tweezers such that the final pellet was ~1 mm in diameter and weighed 2–3 mg.

Surgical Implantation. Thirty-day-old virgin female BALB/c mice (Charles River) were used in all experiments. Mice were anethesized with an i.p. injection of 250–350 μl of avertin [20 mg/ml 2,2,2-tribromoethanol (Aldrich) in saline]. The number 4 inguinal mammary fat pad was surgically exposed, and a 2-mm incision was made through the mammary fat pad outer membrane immediately anterior to the central lymph node. The Elvax pellet was placed within the incision and immobilized under the mammary fat pad outer membrane. Control pellets lacking growth factor were inserted into the contralateral number 4 inguinal mammary fat pad. The wounds were closed using surgical staples, and the mice were allowed to recover under a heat lamp.

To determine the response range and saturation point for each growth factor, mice were implanted with Elvax pellets containing 0.5, 1.0, 2.0, 5.0, 10, or 20 μg of growth factor. In another series of experiments, pellets contained 10 μg of 17 β-estradiol and 1 mg of progesterone (E/P) in addition to growth factor.

Whole-Mount Preparation of Mammary Gland. Mice were sacrificed 3 days following placement of implants. The entire number 4 inguinal mammary fat pad was removed at the nipple and spread onto a precleaned glass slide. The fat pad was air-dried for 10 min and fixed in acidic ethanol (75% ethanol and 25% acetic acid) for 1 h at room temperature. The tissue was incubated in 70% ethanol for 15 min and ddH_2O for 5 min. Ductal structures were stained in carmine solution [0.2% carmine and 0.5% aluminum potassium sulfate (both from Sigma)] for 12–16 h at room temperature. The stained tissue was dehydrated through graded ethanol, defatted in acetone, and cleared in toluene for 12–16 h. The stained and cleared mammary fat pad was mounted under coverslip with Permount (Fisher) and photographed with a slide duplicator.

Histological Examination. For histological examination of mammary gland ductal structures, fat pads were fixed in 4% paraformaldehyde, stained in hematoxylin or carmine solution, dehydrated through graded ethanol into xylene, and cleared in methyl salicylate (Sigma). Ductal structures identified under a dissecting microscope were excised, blocked in paraffin, sectioned at 4 μm, and stained with H&E using standard procedures.

Acknowledgments

We thank John Wysolmerski and Baolin Li for advice on mammary gland whole-mount fixation and staining. We thank Tracey Davison of the Yale University Critical Technologies Program for preparation of paraffin tissue sections. We thank Barbara Vonderhaar for mouse casein antibody and Michael DiGiovanna for advice on immunohistochemical staining of paraffin sections. We thank Don Derby at Focus Chemical Corporation for the generous donation of Evatane 40–55 (Elvax). We also thank Debbie Colditz for technical support and other members of the Stern laboratory for their advice and critical insights.

References

1. Daniel, C. W., and Silberstein, G. B. Postnatal development of the rodent mammary gland. *In:* M. C. Neville and C. W. Daniel (eds.), The Mammary Gland: Development, Regulation, and Function, pp. 3–36. New York: Plenum Publishing Corp., 1987.

2. Russo, J., and Russo, I. H. Development of the human mammary gland. *In:* M. C. Neville and C. W. Daniel (eds.), The Mammary Gland: Development, Regulation, and Function, pp. 67–93. New York: Plenum Publishing Corp., 1987.

3. Topper, Y. J., and Freeman, C. S. Multiple hormone interactions in the developmental biology of the mammary gland. Physiol. Rev., *60:* 1049–1106, 1980.

4. Vonderhaar, B. K. Hormones and growth factors in mammary gland development. *In:* C. M. Veneziale (eds.), Control of Cell Growth and Proliferation, pp. 11–33. Princeton, NJ: van Nostrand-Reinhold, 1984.

5. Dembinski, T. C., and Shiu, R. P. C. Growth factors in mammary gland development and function. *In:* M. C. Neville and C. W. Daniel (eds.), The Mammary Gland: Development, Regulation, and Function, pp. 355–381. New York: Plenum Publishing Corp., 1987.

6. Bates, S. E., Davidson, N. E., Valverius, E. M., Freter, C. E., Dickson, R. B., Tam, J. P., Kudlow, J. E., Lippman, M. E., and Salomon, D. S. Expression of transforming growth factor α and its messenger ribonucleic acid in human breast cancer: its regulation by estrogen and its possible functional significance. Mol. Endocrinol., *2:* 543–555, 1988.

7. Salomon, D. S., Kidwell, W. R., Kin, N., Ciardiello, F., Bates, S. E., Valverius, E. M., Lippman, M. E., Dickson, R. B., and Stampfer, M. R. Modulation by estrogen and growth factors of transforming growth factor-α and epidermal growth factor expression in normal and malignant human mammary epithelial cells. Recent Results Cancer Res., *113:* 57–69, 1989.

8. Hiramatsu, M., Kashimata, M., Takayama, F., and Minami, N. Developmental changes in and hormonal modulation of epidermal growth factor concentration in the rat submandibular gland. J. Endocrinol., *140:* 357–363, 1994.

9. Martinez-Lacaci, I., Saceda, M., Plowman, G. D., Johnson, G. R., Normanno, N., Salomon, D. S., and Dickson, R. B. Estrogen and phorbol esters regulate amphiregulin expression by two separate mechanisms in human breast cancer cell lines. Endocrinology, *136:* 3983–3992, 1995.

10. Mouihate, A., and Lestage, J. Estrogen increases the release of epidermal growth factor from individual pituitary cells in female rats. J. Endocrinol., *146:* 495–500, 1995.

11. Salomon, D. S., Brandt, R., Ciardiello, F., and Normanno, N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit. Rev. Oncol., *19:* 183–232, 1995.

12. Snedeker, S. M., Brown, C. F., and DiAugustine, R. P. Expression and functional properties of transforming growth factor α and epidermal growth factor during mouse mammary gland ductal morphogenesis. Proc. Natl. Acad. Sci. USA, *88:* 276–280, 1991.

13. Liu, S. C., Sanfilippo, B., Perroteau, I., Derynck, R., Salomon, D. S., and Kidwell, W. R. Expression of transforming growth factor α (TGFα) in differentiated rat mammary tumors: estrogen induction of TGFα production. Mol. Endocrinol., *1:* 683–692, 1987.

14. Smith, J. A., Barraclough, R., Fernig, D. G., and Rudland, P. S. Identification of α transforming growth factor as a possible local trophic agent for the mammary gland. J. Cell. Physiol., *141:* 362–370, 1989.

15. Liscia, D. S., Merlo, G., Ciardiello, F., Kim, N., Smith, G. H., Callahan, R., and Salomon, D. S. Transforming growth factor-α messenger RNA: localization in the developing adult rat and human mammary gland by *in situ* hybridization. Dev. Biol., *140:* 123–131, 1990.

16. Panico, L., D'Antonio, A., Salvatore, G., Mezza, E., Tortora, G., DeLaurentiis, M., DePlacido, S., Giordano, T., Merino, M., Salomon, D. S., Gullick, W. J., Pettinato, G., Schnitt, S. J., Bianco, A. R., and Ciardiello, F. Differential immunohistochemical detection of transforming growth factor α, amphiregulin, and cripto in human normal and malignant breast tissues. Int. J. Cancer, *65:* 51–56, 1996.

17. Qi, C-F., Liscia, D. S., Normanno, N., Merlo, G., Johnson, G., Gullick, W. J., Ciardiello, F., Saeki, T., Brandt, R., Kim, N., Kenney, N., and Salomon, D. S. Expression of transforming growth factor α, amphiregulin, and cripto-1 in human breast carcinomas. Br. J. Cancer, *69:* 903–910, 1994.

18. Kenney, N. J., Huang, R-P., Johnson, G. R., Wu, J-X., Okamura, D., Matheny, W., Kordon, E., Gullick, W. J., Plowman, G., Smith, G. H., Salomon, D. S., and Adamson, E. D. Detection and localization of amphiregulin and cripto-1 expression in the developing postnatal mouse mammary gland. Mol. Reprod. Dev., *41:* 277–286, 1995.

19. Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, K. M., Birchmeier, C., and Birchmeier, W. Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J. Cell Biol., *131:* 215–226, 1995.

20. Coleman, S., Silberstein, G. B., and Daniel, C. W. Ductal morphogenesis in the mouse mammary gland: evidence supporting a role for epidermal growth factor. Dev. Biol., *127:* 304–315, 1988.

21. Gullick, W. J. The role of the epidermal growth factor receptor and the c-erbB-2 protein in breast cancer. Int. J. Cancer, *5* (Suppl.)*:* 55–61, 1990.

22. Press, M. F., Cordon-Cardo, C., and Slamon, D. J. Expression of the *Her-2/neu* proto-oncogene in normal adult and fetal tissues. Oncogene, *5:* 953–962, 1990.

23. Manning, D. L., Nicholson, R. I., and Eaton, C. L. Role of oestrogens and growth factors in the aetiology of breast cancer. Recent Adv. Endocrinol. Metab., *27:* 133–149, 1992.

24. Hynes, N. E., and Stern, D. F. The biology of *erbB-2/neu/HER-2* and its role in cancer. Biochim. Biophys. Acta, *1198:* 165–184, 1994.

25. Dickson, R. B., and Lippman, M. E. Growth factors in breast cancer. Endocr. Rev., *16:* 559–589, 1995.

26. Lee, D. C., Fenton, S. E., Berkowitz, E. A., and Hissong, M. A. Transforming growth factor α: expression, regulation, and biological activities. Pharmacol. Rev., *47:* 51–85, 1995.

27. Nguyen, B., Keane, M. M., and Johnston, P. G. The biology of growth regulation in normal and malignant breast epithelium: from the bench to clinic. Crit. Rev. Oncol. Hematol., *20:* 223–236, 1995.

28. Lu, H. S., Chang, D., Philo, J. S., Zhang, M., Sun, J., Wen, J., Yanagihara, D., Karunagaran, D., Yarden, Y., and Ratzkin, B. Studies on the structure and function of glycosylated and nonglycosylated *neu* differentiation factors. J. Biol. Chem., *270:* 4784–4791, 1995.

29. Marikovsky, M., Lavi, S., Pinkas-Kramarski, R., Karunagaran, D., N. L., Wen, D., and Yarden, Y. ErbB-3 mediates differential mitogenic effects of NDF/heregulin isoforms on mouse keratinocytes. Oncogene, *10:* 1403–1411, 1995.

30. Marte, B. M., Graus-Porta, D., Jeschke, M., Fabbro, D., Hynes, N. E., and Taverna, D. NDF/heregulin activates MAP kinase and p70/p85 S6 kinase during proliferation or differentiation of mammary epithelial cells. Oncogene, *10:* 167–175, 1995.

31. Ram, T. G., Kokeny, K. E., Dilts, C. A., and Ethier, S. P. Mitogenic activity of neu differentiation factor/heregulin mimics that of epidermal growth factor and insulin-like growth factor-I in human mammary epithelial cells. J. Cell. Physiol., *163:* 589–596, 1995.

32. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben Levy, R., and Yarden, Y. Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. Cell, *69:* 205–216, 1992.

33. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Koski, R. A., Lu, H. S., and Yarden, Y. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell, *69:* 559–572, 1992.

34. Bacus, S. S., Gudkov, A. V., Zelnick, C. R., Chin, D., Stern, R., Stancovski, I., Peles, E., Ben Baruch, N., Farbstein, H., Lupu, R., Wen, D., Sela, M., and Yarden, Y. Neu differentiation factor (heregulin) induces expression of intracellular adhesion molecule 1: implication for mammary tumors. Cancer Res., *53:* 5251–5261, 1993.

35. Culouscou, J. M., Plowman, G. D., Carlton, G. W., Green, J. M., and Shoyab, M. Characterization of a breast cancer cell differentiation factor that specifically activates the HER4/p180^{erbB4} receptor. J. Biol. Chem., *268:* 18407–18410, 1993.

36. Mudge, A. W. New ligands for neu? Curr. Biol., *3:* 361–364, 1993.

37. Vonderhaar, B. K. Local effects of EGF, α-TGF, and EGF-like growth factors on lobuloalveolar development of the mouse mammary gland *in vivo*. J. Cell. Physiol., *132:* 581–584, 1987.

38. Tonelli, Q. J., and Sorof, S. Epidermal growth factor requirement for development of cultured mammary gland. Nature (Lond.), *285:* 250–252, 1980.

39. Lydon, J. P., DeMayo, F. J., Funk, C. R., Mani, S. K., Hughes, A. R., Montgomery, C. A., Jr., Shyamala, G., Conneely, O. M., and O'Malley, B. W. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev., *9:* 2266–2278, 1995.

40. Saeki, T., Cristiano, A., Lynch, M. J., Brattain, M., Kim, N., Normanno, N., Kenney, N., Ciardiello, F., and Salomon, D. S. Regulation by estrogen through the 5′-flanking region of the transforming growth factor α gene. Mol. Endocrinol., *5:* 1955–1963, 1991.

41. Normanno, N., Qi, C-F., Gullick, W. J., Persico, G., Yarden, Y., Wen, D., Plowman, G., Kenney, N., Johnson, G., Kim, N., Brandt, R., Martinez-Lacaci, I., Dickson, R. B., and Salomon, D. S. Expression of amphiregulin, cripto-1, and heregulin α in human breast cancer cells. Int. J. Oncol., *2:* 903–911, 1993.

42. Stern, D. F., and Kamps, M. P. EGF-stimulated tyrosine phosphorylation of p185^{neu}: a potential model for receptor interactions. EMBO J., *7:* 995-1001, 1988.

43. Goldman, R., Levy, B. R., Peles, E., and Yarden, Y. Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. Biochemistry, *29:* 11024–11028, 1990.

44. Wada, T., Qian, X., and Greene, M. I. Intermolecular association of the p185^{neu} protein and EGF receptor modulates EGF receptor function. Cell, *61:* 1339–1347, 1990.

45. Earp, H. S., Dawson, T. L., Li, X., and Yu, H. Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. Breast Cancer Res. Treat., *35:* 115–132, 1995.

46. Sliwkowski, M., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J. Biol. Chem., *269:* 14661–14665, 1994.

47. Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and Yarden, Y. ErbB-3 and erbB-4 function as respective low and high affinity receptors of all *neu* differentiation factor/heregulin isoforms. J. Biol. Chem., *269:* 25226–25233, 1994.

48. Riese, D. J., II, van Raaij, T. M., Plowman, G. D., Andrews, G. C., and Stern, D. F. Cellular response to neuregulins is governed by complex interactions of the erbB receptor family. Mol. Cell. Biol., *15:* 5770–5776, 1995.

49. DiFiore, P. P., Segatto, O., Taylor, W. G., Aaronson, S. A., and Pierce, J. H. EGF receptor and *erb*B-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. Science (Washington DC), *248:* 79–83, 1990.

50. Riese, D. J., II, Bermingham, Y., Raaij, T. M. v., Buckley, S., Plowman, G. D., and Stern, D. F. Betacellulin activates the epidermal growth factor receptor and erbB-4 and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-β. Oncogene, *12:* 345–353, 1996.

51. Zhang, K., Sun, J., Liu, N., Wen, D., Chang, D., Thomason, A., and Yoshinaga, S. K. Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. J. Biol. Chem., *271:* 3884–3890, 1996.

52. Lee, K-F., Simon, H., Chen, H., Bates, B., Hung, M-C., and Hauser, C. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature (Lond.), *378:* 394–398, 1995.

53. Meyer, D., and Birchmeier, C. Multiple essential functions of neuregulin in development. Nature (Lond.), *378:* 386–390, 1995.

54. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W. J., Wood, W. I., Goeddel, D. V., and Vandlen, R. L. Identification of heregulin, a specific activator of p185^{erbB2}. Science (Washington DC), *256:* 1205–1210, 1992.

55. Sambrook, S., Fritsch, E. F., and Maniatis, T. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.

56. Barbacci, E. G., Guarino, B. C., Stroh, J. G., Singleton, D. H., Rosnack, K. J., Moyer, J. D., and Andrews, G. C. The structural basis for the binding specificity of epidermal growth factor and heregulin binding. J. Biol. Chem., *270:* 9585–9589, 1995.

57. Silberstein, G. B., and Daniel, C. W. Reversible inhibition of mammary gland growth by transforming growth factor-β. Science (Washington DC), *237:* 291–293, 1987.